CHARACTERISATION OF HETEROLOGOUS PRIME-BOOST VACCINATION STRATEGIES:

AN INVESTIGATION INTO THE NATURE AND DELIVERY OF VACCINES AND THE SUBSEQUENT GENERATION OF IMMUNE RESPONSES

Benjamin James Searle

Infection and Immunity

Submitted to the University of Glasgow

for the degree of PhD

September, 2004

ACKNOWLEDGEMENTS

There are many people without whom this thesis would never have been completed in anything like its current form, and others without whom the work that went into it would have been much more difficult. I am indebted to R. Schirmbeck, J. Brewer and MedevaPharma Plc for providing essential cell-lines and reagents. Just as importantly, there were those who made my time here enjoyable.

The people in the department, too many to mention individually, but especially people in the offices I've worked in, climbing mates and pub mates, have proven themselves good friends and have made the time I have spent here all the more pleasant. In the same vein, I would like to acknowledge the fine pubs in the west end of Glasgow and the many cheerful hours spent in them.

I would like to thank all of those in Infection and Immunity who were ready to lend reagents, use of equipment, advice and moral support. Their generosity was invaluable. In the laboratory, I would like to specifically thank Moira, Eoin, Irene and Denise for their able technical assistance. Also Mihaela for helping introduce me to the lab and doing the groundwork which this thesis continued.

Many thanks should go to Rosie, who is responsibly for very much of what I've learned about the mysteries of immunology, practical lab work, salvaging experiments, crossword puzzles, nipping out for coffee, eating, drinking and being merry. It wouldn't have been the same without her.

Also especial thanks have to go to Gill, for being a great supervisor, displaying superhuman patience, for unfailingly constructive criticism in liberal quantities, for being willing to get her hands dirty in the lab, for providing more experience of childcare than is common in most PhD projects, but most of all, for all the whisky. Cheers

Finally, to my Mum and Dad, for their constant support, and without whom I couldn't have done this.

ABSTRACT

To date, no single, ideal strategy of vaccination has been identified that can provide complete protection against every form of viral, bacterial and parasitic disease encountered currently by man. This is because every pathogen requires activation of a unique immunological response to ensure effective clearance from the body. Thus, one of the main challenges of modern vaccine development is to devise strategies of vaccination that can be tailored to ensure the rapid and effective removal of a pathogen before it is able to initiate infection. In addition, the concept of therapeutic vaccines to treat established disease has recently been introduced for the treatment of chronic infections such as Hepatitis B virus (HBV).

This thesis describes work undertaken to consider how strategies of vaccination can be manipulated to generate specific types and magnitude of immune response to prevent infection or treat established infection. In particular, the project considered the combined use of naked DNA vaccination with a second form of vaccination to maximise the specific immune response generated.

The vaccination strategies employed were based on the initial delivery of DNA vaccine through intramuscular injection or ballistic delivery using a gene gun, followed by heterologous boosts, based on the same antigen delivered using an alternative route. These boost strategies included a) intramuscular delivery of purified recombinant HBcAg, b) mucosal delivery of HBcAg expressed by an attenuated strain of *Salmonella typhimurium*, and c) intranasal delivery of the protein accompanied by a mucosal adjuvant.

Following each vaccination regime tested a number of specific immune responses, including serum and mucosal antibody production, $CD4^+$ T helper

proliferation in spleens and lymph nodes, $CD8^+$ T cell activation and killing were measured. These experiments revealed that the character of the immune responses primed in response to DNA vaccination differed according to route of immunisation, with intramuscular vaccination inducing a rapid CTL response. $CD4^+$ T cells generated appeared to be of the Th1 phenotype and showed the strongest localisation in the spleen. In contrast, following vaccination with the gene gun, $CD4^+$ cells of a Th2 phenotype were generated with responses being found to be stronger in the local draining lymph nodes than the spleen.

Combining DNA delivered using the intramuscular route with recombinant purified protein delivered by the same route was effective at boosting systemic antibody titres, as was boosting using attenuated *Salmonella* expressing the same antigen when delivered to the mucosal surface of the gut. In contrast, boosting of DNA primed animals with purified protein, even in the presence of an appropriate mucosal adjuvant, was not successful at increasing titres of systemic antibodies. Interestingly, specific mucosal immune responses were absent when either of the heterologous vaccination strategies used a mucosal boosting approach. The implications of the characterisation of the immune responses to the various prime/boost protocols employed are discussed and their potential use for the development of new prophylactic and therapeutic vaccine design considered.

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ABBREVIATIONS

APC	Antigen presenting cells
APS	Ammonium persulphate
BCR	B cell receptor
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
CFU	Colony forming units
CLN	Cervical lymph nodes
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
CpG	Cytidine-phosphate-guanisine dinucleotides
CPM	Counts per minute
DC	Dendritic cells
EGTA	Ethylene glycol tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
EU	Endotoxin units
FCS	Foetal calf serum
GM-CSF	Granulocyte-monocyte colony stimulating factor
IFN	Interferon
Ig	Immunoglobulin
i.g.	Intra-gastric
IL	Interleukin
i.m.	Intra-muscular
i.n.	Intra-nasal

IPTG	Isopropyl-β-D-Thiogalactopyranoside
LAL assay	Limulus amoebocyte lysate assay (LPS quantification)
LC	Langerhan's Cell
LPS	Lipopolysaccharide = endotoxin
LT	*E. coli* heat-labile enterotoxin
MACS	Magnetic associated cell sorting
MHC	Major histocompatibility complex
NCS	Newborn calf serum
OD	Optical density
OPD	O-phenyl diamine
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PP	Peyer's patches
PVP	Polyvinyl pyrrolidone
s.c.	Sub-cutaneous
TEMED	Tetra methyl ethylene diamine
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor

DECLARATION

I declare that the work described within this thesis has not been accepted in any previous publication for a higher degree and that unless otherwise stated all work has been carried out by myself and that all sources of information have been acknowledged.

1 INTRODUCTION

1.1 Aims of vaccination

The aim of vaccination is to provide a human or animal with immunity to a specific disease. Traditionally this has been achieved by exposing the immune system to non-pathogenic components (antigens) of a disease-causing organism, or to an avirulent form of the entire pathogen. This exploits the immune system's capacity to effect a more rapid and potent memory response to foreign antigens on subsequent re-exposure to the antigens during an actual infection. An effective vaccine should provide long lasting, protective immunity against the disease and where possible reduce the capacity of the organism to spread to other susceptible hosts.

1.2 History of Vaccination

1.2.1 Development of Vaccination

The idea of deliberate exposure of an individual to a relatively harmless form of disease in order to protect against the full virulence of a natural infection is not new. The process of variolation, a term given to the practice of scoring the skin and deliberately exposing it to material from the pustules on the skin of a smallpox victim, was reported to have been used as far back as the 17th century (Bazin 2003). Variolation provoked a very mild form of the smallpox and protected the recipient when exposed to the fully virulent form of the disease. In 1796 Edward Jenner took the process a step further, by testing the commonly held idea that infection

with cowpox provided protection from smallpox. This was confirmed when he deliberately inoculated a young boy with material from the pustules on the skin of an infected cow. In order to confirm the protective effects of the treatment the boy was later exposed to an attenuated strain of smallpox. The boy did not display any disease symptoms, and the process of vaccination (from Vaccinia, the virus that causes cowpox) was born (Baxby 2002; Bazin 2003). Jenner's vaccination had the great advantage of providing lasting protection without risk of the vaccine recipient becoming the source of a smallpox outbreak.

1.2.2 The Requirement for New Vaccines

As a result of this early work, vaccine development against a variety of diseases has continued for more than 200 years. Over this period of time there has been a dramatic increase in the level of understanding of both pathogenesis and the nature of the immune system as well as improvements in public hygiene and successful treatment of disease by chemotherapy. Despite these advances, there remain many infections for which there are no effective vaccines. These include malaria, HIV, hepatitis C virus, anthrax, and many others.

In many older vaccine formulations, protection is mediated largely by the induction of strong antibody (humoral) responses. Without denying the undoubted success of such vaccines, humoral responses alone are not always sufficient to offer protection. Observations in human patients and animal models of disease show that other processes including responses mediated by specific cells of the body are also required. Thus much current vaccine research focuses on the generation of broad and effective immune responses (Plotkin 2003).

In addition to diseases that have been present in human populations for a long period of time, there are also newly emerging infections such as HIV and SARS, which provide an ongoing challenge to vaccinologists. These pathogens are often highly virulent and reinforce the need for continued research to identify vaccine strategies that provide wider application.

Immunity to a disease often involves the activation of a combination of different effector mechanisms. The protective mechanisms required may be largely dependent on the pathogen in question and the causes of its pathogenesis and its lifestyle. The way in which these mechanisms are activated and how they function to protect against disease are described below.

1.3 Innate Immunity

The immune system can be split into two separate but linked entities; the innate and the specific (or adaptive) immune systems. Innate immunity is based upon activation of responses following tissue damage or recognition of types of molecules particularly associated with pathogens. The defence mechanisms that make up innate immunity are able to act immediately, because unlike specific immunity they do not rely on the clonal expansion of cells.

The innate defence mechanisms include mechanical systems, the action of cells such as phagocytes and the complement system. The innate immune system has no memory mechanism of its own, however, it can still have relevance in vaccine development. Although the innate and specific systems are in many ways separate and are induced by different mechanisms, each can enhance the other to provide maximum effectiveness (Medzhitov and Janeway Jr. 1998). For example, cytokines secreted by innate cells (e.g. macrophages) and specific cells (such as

lymphocytes) can increase the efficacy of both cell types (Biron 1998). Also, molecules found associated with the invading pathogen itself can have a significant impact on non-specific activation of the immune system. A number of these pathogen-associated molecular patterns, or PAMPs have been recently identified and play an important role in the activation of naïve lymphocytes (see section 1.4.1, Antigen Processing and Lymphocyte Activation).

Physically, the body is protected from infection by the epithelial barriers of the skin and mucosal surfaces of the intestines, lungs and urogenital tracts. Pathogens can initiate an infection only when these surfaces are colonised or crossed. In particular, the normal fauna of the skin and mucosal surfaces can provide a considerable defence by competing for space and nutrients with potential pathogens. Secretion of mucus is also important, coating micro-organisms and preventing them from adhering to the surface and when combined with the co-ordinated beating of cilia on the surface of epithelial cells in the lungs, allows expulsion of microbes from these surfaces.

In addition to mechanical barriers at the epithelia, there are also chemical products and degradative proteins that can kill or inhibit the growth of bacteria. These include lysozyme, present in saliva and tears, which can cause the lysis of bacteria, acid pH in the stomach and digestive enzymes that destroy the majority of pathogens prior to their arrival in the gut.

1.3.1 Non-specific Activation of Immune Cells

A range of phagocytic cells that are able to recognise, ingest and kill many pathogens that do penetrate the epithelia include macrophages, which are found in many tissues of the body and are highly efficient phagocytes, and neutrophils that

are present in the blood. These can quickly penetrate tissues hosting an incipient infection and phagocytose the organisms present.

A number of these cells have been shown to be activated by PAMPs, which stimulate Toll-like receptors (TLRs) found on the surface of some immune system cells. This leads to the activation of signalling pathways that result in the production of antimicrobial products and inflammatory cytokines by these cells. In addition, stimulation of TLRs triggers dendritic cell (DC) maturation and results in the induction of co-stimulatory molecules and increased antigen-presenting capacity (Krug *et al.* 2001). Thus, microbial recognition by TLRs helps to directly activate innate immune responses for immediate killing of pathogens and indirectly stimulate adaptive responses (Janeway and Medzhitov 2002).

Stimulation of TLRs via PAMPs provides an early warning signal to the immune system that pathogens are present, thus allowing the rapid deployment of innate defence mechanisms based on a general molecular pattern, rather than specific antigen recognition (Janeway and Medzhitov 2002).

To date, several PAMPs have been identified. Two of these; bacterial lipopolysaccharide (LPS) and unmethylated Cytidine-phosphate-guanisine dinucleotides (CpG motifs) in DNA (Brown and Corral 2002; Janeway and Medzhitov 2002; Royle *et al.* 2003), have direct relevance to this project.

Gram negative bacteria such as *E. coli* and *Salmonella* carry endotoxin, or LPS as an integral part of their outer membrane. The structure of LPS, as shown in Figure 1-1, consists of highly conserved lipid A, which anchors the structure into the cell wall, the core polysaccharide and the hyper-variable O-polysaccharide. This antigen is highly immunogenic and is readily recognised by antibodies. It is the variation in the recognition of this region by antibodies that results in the many different serotypes of *Salmonella* (Ewing 1972). It can also have a significant effect

on the biology of the organism; *Salmonella* can alter the nature of the LPS molecule it expresses under different conditions. This ability allows the bacteria to change its surface characteristics to suit the environment of gut epithelial cells or protect itself against the intracellular killing mechanisms within the phagosomes of macrophages (Ernst *et al.* 1999; Freudenberg *et al.* 2001).

However, LPS can also interact with the innate immune system, directly through TLR-4 found on the surface of DCs and macrophages (Royle *et al.* 2003). Stimulation through TLR-4 signalling by LPS causes upregulation of macrophage killing mechanisms, increased cytokine production and higher levels of co-stimulatory molecules on the surface of APCs. The presence of these molecules, along with the microbial antigens presented by macrophages and DCs, helps activate $CD4^+$ Th cells leading to a co-ordinated adaptive immune response (Ernst *et al.* 2001; Royle *et al.* 2003).

The CpG motifs found in bacterial DNA are another example of a PAMP. These unmethylated CpG motifs found in a particular nucleotide context in bacterial but not eukaryotic DNA activate the immune system via TLR–9. They are discussed in more detail in the section on DNA vaccination below (section 1.5.4.3).

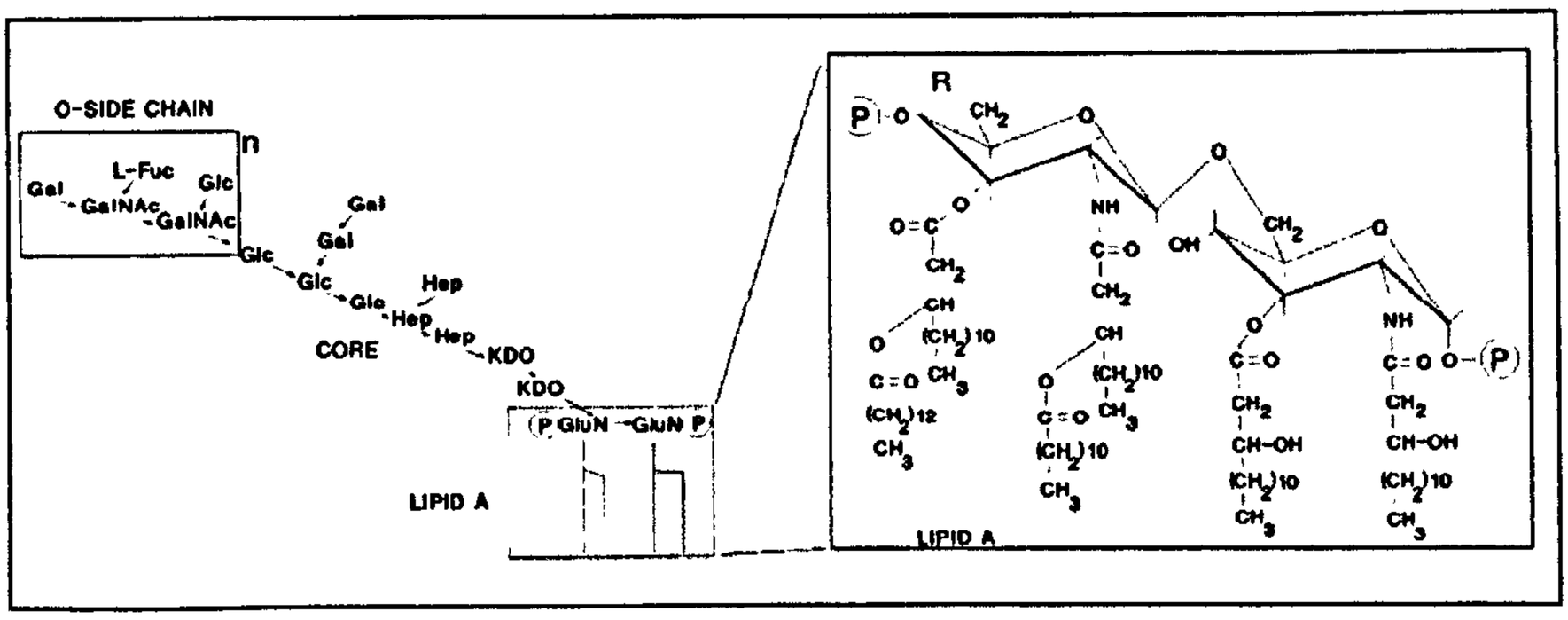


Figure 1-1: Composition of bacterial LPS

Lipid A, the mitogenic region of the LPS molecule, is made up of a variable number and chain lengths of fatty acid. These are linked to the core region of LPS by phosphorylated diglucosamine (GluN) and 2-keto-3-deoxymanno-octonic acid (KDO). The O-side chain polysaccharide or O-antigen consists of repeating oligosaccharide units. These also vary between strains and species; the O86 antigen of *E. coli* is shown here. This variation can have a large impact on phenotype and pathogenicity, for example bacteria with complete O antigen form smooth colonies whereas bacteria which cannot synthesise the antigen form rough colonies. Figure adapted from 'Immunological aspects of bacterial virulence' (Patrick and Larkin 1995).

1.3.2 Chemokines and Inflammation

Once activated via the TLRs, cells such as macrophages are stimulated to secrete several cytokines including inflammatory mediators. These include two sub-families of chemokines, α and β, which can cause an influx of cells. The α-chemokines, such as interleukin-8 (IL-8), attract neutrophils, whereas the β-chemokines attract macrophages (Herbert *et al.* 1995). These molecules help target neutrophils and macrophages to the site of infection by up-regulating expression of leukocyte adhesion molecules in the endothelial cells of nearby blood vessels. As well as monocytes and neutrophils, specific immune cells such as lymphocytes migrate to the site of inflammation. The inflammatory response can therefore speed the genesis of a specific immune response (Biron 1998).

1.3.3 Complement

Complement is another molecular component of the innate immune system. This system is made up of a large number of proteins present in the plasma that respond to the presence of microbes by the binding of certain proteins to the pathogen surface. Following activation, they can function in a number of ways, including opsonisation of pathogens which enhances the efficiency with which phagocytes such as macrophages are able to internalise them. Complement is regarded as part of the innate immune system, however, the ability to induce an inflammatory response is also an example of how the innate and specific immune systems can communicate. Complement can also act as an effector arm of specific immunity, as it can additionally be activated by the formation of antibody-antigen complexes (Barrington *et al.* 2001).

1.4 Antigen-Specific Immunity

1.4.1 Antigen Processing and Lymphocyte Activation

Specific or adaptive immunity is based on the precise recognition by lymphocytes of antigen-fragments containing specific epitopes. The antigens of a micro-organism are processed by cells and the resulting peptide fragments are presented on the cell surface in the context of Major Histocompatibility (MHC) molecules. These peptides are subject to surveillance by T lymphocytes. If the T cell receptor (TCR) specifically recognises the epitope contained in the fragments displayed on the surface of antigen presenting cells (APCs), along with an appropriate secondary signal, the T cells are activated. They then proliferate and differentiate into effector cells with a range of functions including the enhancement of antibody production by B cells, phagocytosis by macrophages and activation of cytotoxicity.

1.4.1.1 Endogenous Antigen Processing and $CD8^+$ T cell activation

Antigens expressed within most body cells can be processed through the endogenous pathway, illustrated in Figure 1-2. Although this pathway is utilised by both professional and non-professional APCs, it can be modulated in professional APCs (such as DCs) by an increased expression of co-stimulatory molecules. The antigens processed through this pathway are largely drawn from defective ribosomal products (Lehner and Cresswell 2004). The pathway is therefore open to any protein expressed in the cytosol, including self-antigen, virus antigen and DNA vaccine antigens. However, proteins from sources other than the cytosol are also able to enter the pathway, as discussed below in section 1.4.1.3, Cross Presentation.

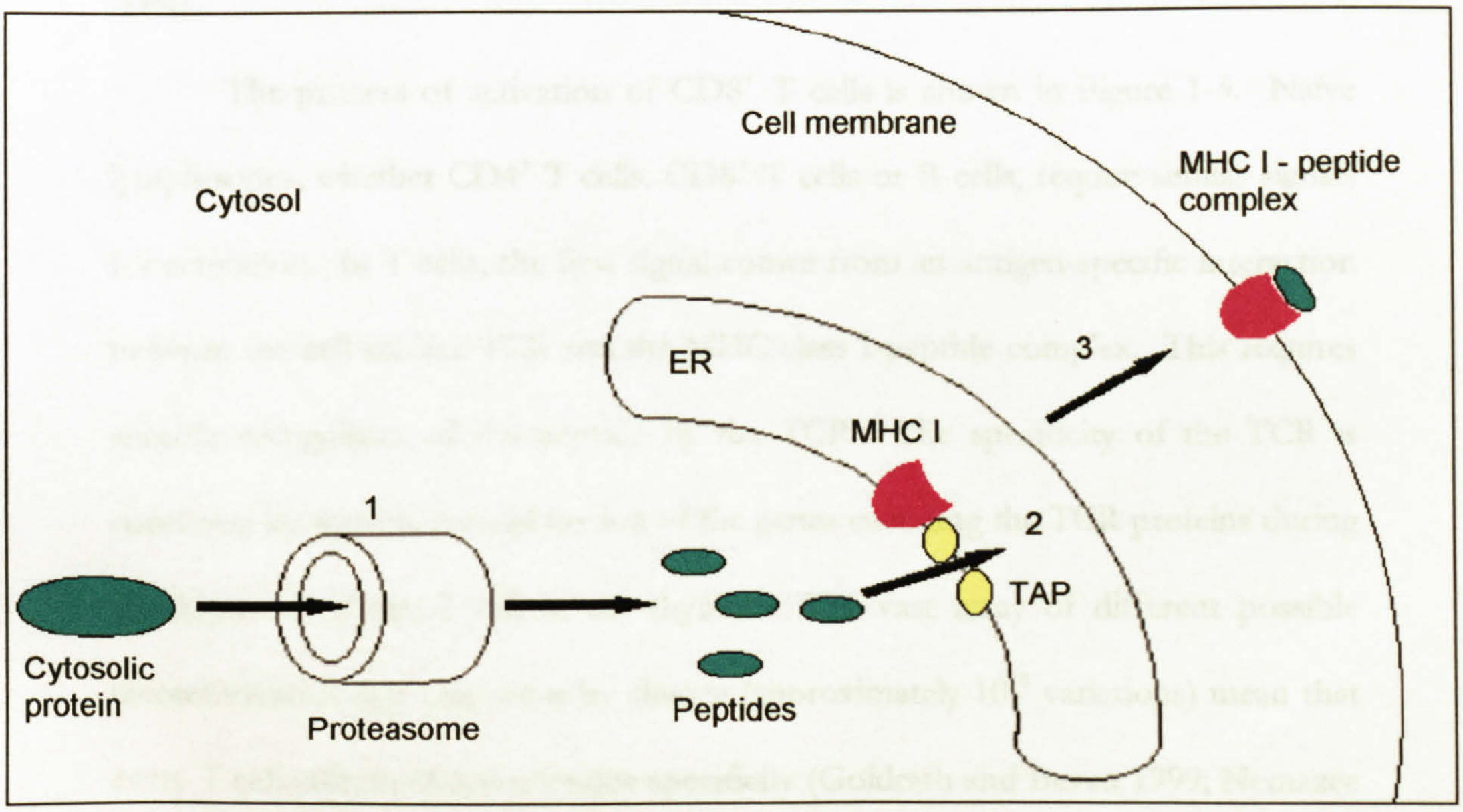


Figure 1-2: The endogenous antigen presentation pathway

Recently synthesised ribosomal products are degraded by proteasomes (1) and peptidases. The resulting peptidases are transported into the ER by TAP molecules (2). In the ER peptides are able to bind to MHC class I molecules. The MHC-peptide complex is then able to migrate to the cell surface (3).

The antigens are broken down by proteasomes into 8 – 10 amino acid peptide fragments, which enter the endoplasmic reticulum (ER) via the transporter associated with antigen-processing (TAP) molecule. Associated with TAP are MHC class I molecules to which some of the peptides will be able to bind. On binding, the three-dimensional structure of MHC I is altered resulting in the release of the peptide-MHC complex from associated ER proteins. The complex then migrates to the cell surface via the Golgi apparatus where it is exposed to $CD8^+$ T cell surveillance (Heemels and Ploegh 1995; Williams *et al.* 1996; Lehner and Cresswell 2004).

The process of activation of $CD8^+$ T cells is shown in Figure 1-3. Naïve lymphocytes, whether $CD4^+$ T cells, $CD8^+$ T cells or B cells, require similar signals for activation. In T cells, the first signal comes from an antigen-specific interaction between the cell surface TCR and the MHC class I-peptide complex. This requires specific recognition of the peptide by the TCR. The specificity of the TCR is conferred by somatic recombination of the genes encoding the TCR proteins during development of the T cell in the thymus. The vast array of different possible recombinations that can occur by chance (approximately 10^{18} variations) mean that every T cell effectively has a unique specificity (Goldrath and Bevan 1999; Nemazee 2000). This variation is key to the T cell population and provides the capacity to respond specifically to a huge range of different antigens (Correia-Neves *et al.* 2001). Such wide variety ensures that some cells will be generated bearing TCRs that are specific for self-antigens. Usually, cells that bind to self-antigens during development undergo apoptosis, preventing autoimmunity (Davis and Bjorkman 1988; Oltz 2001). As well as direct TCR-peptide interactions between the T cell and the APC, the $CD8^+$ molecule binds to invariant regions of MHC I in an antigen-independent fashion (Garboczi *et al.* 1996).

The TCR-peptide specific interaction along with $CD8^+$-MHC binding is sufficient to trigger previously activated $CD8^+$ T cells. However, naïve lymphocytes require an additional, secondary, signal in order to initiate proliferation and differentiation into effector cells. This is provided by a second cognate interaction between a co-stimulatory molecule on the APC and its co-receptor on the naïve T cell. The level of expression of APC co-stimulatory molecules varies depending on the activation status of these cells and whether the APC is a 'professional' presenter. For example, a DC is a professional APC and is characterised by constitutive expression of co-stimulatory molecules such as the B7 family members CD80 and CD86 (which bind to CD28 on the T cell), or CD40 (which binds to CD40 ligand). Expression is increased even further on activation of the cell. However, many other cells expressing MHC class I need to upregulate the surface expression of these molecules whilst processing exogenous antigens. Expression of co-stimulatory molecules is tightly regulated by signalling events associated with the recognition of molecules associated with tissue damage or with certain pathogens (PAMPs) by receptors including the TLRs (Harding *et al.* 1992; Gonzalo *et al.* 2001; Shedlock and Shen 2003).

The co-stimulatory requirement for activation of naïve $CD8^+$ T cells are higher than that required for naïve $CD4^+$ cells. This ensures that the potentially devastating cytotoxic response is not induced inappropriately. To activate a naïve $CD8^+$ cell requires either antigen presentation by a DC, which naturally express high level of co-stimulatory molecules, or the action of Th cells with the same antigen-specificity, that can upregulate co-stimulatory molecule expression in other types of APC.

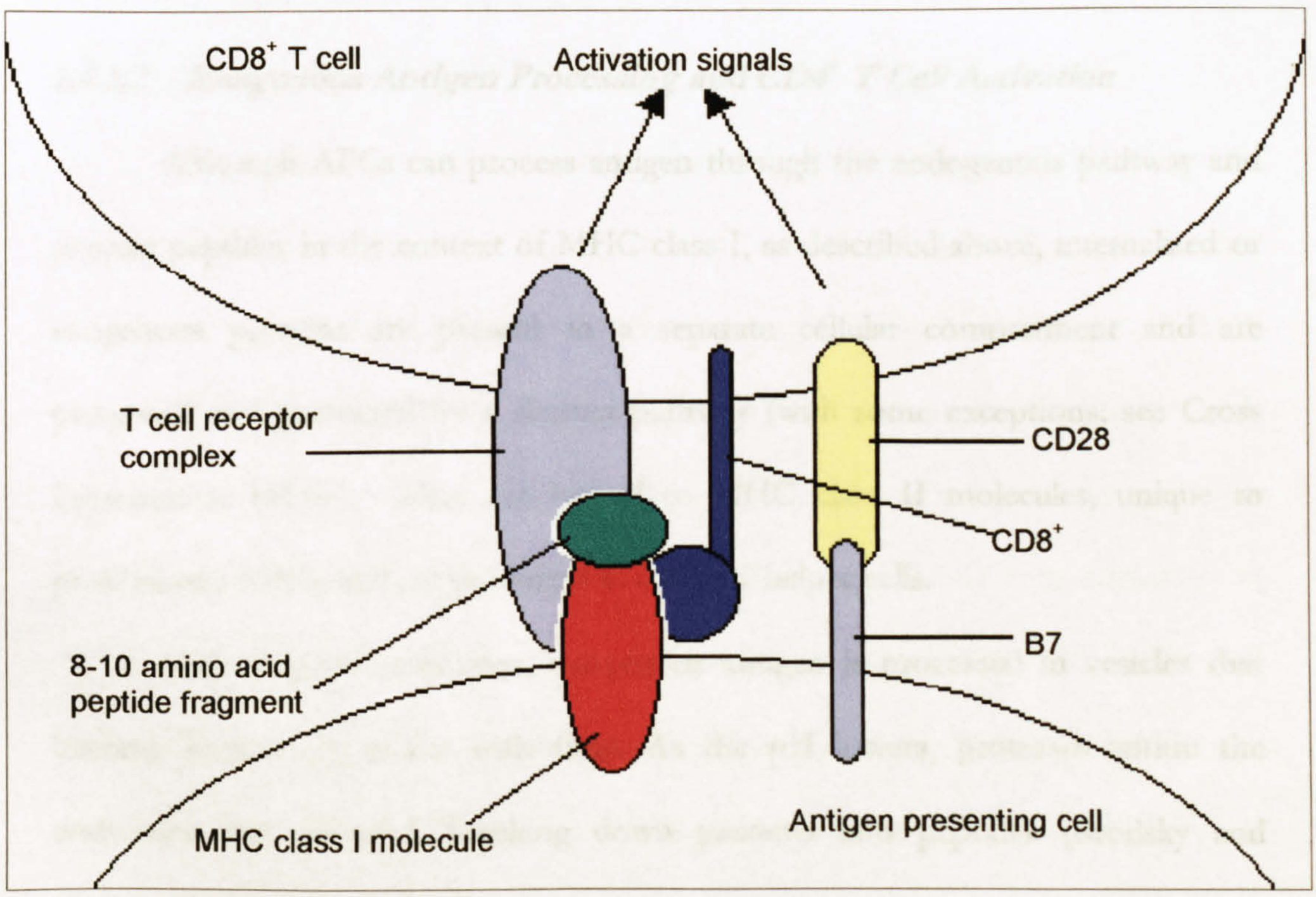


Figure 1-3: CD8$^+$ T cell antigen recognition and activation

Peptides in the context of MHC I are specifically recognised by the TCR and CD8$^+$ binds to MHC I. This is sufficient for activation of effector CD8$^+$ T cells. An additional signal is required for activation of naïve cells, to initiate proliferation and differentiation. This comes from co-stimulatory molecules expressed by the APCs such as B7, which binds to CD28 on the T cell, providing the secondary signal.

The requirement for a secondary signal provides an additional level of regulation in the development of a primary immune response, and helps to ensure that responses only develop against pathogens or antigens associated with tissue pathology, what is referred to as a 'danger' signal. A successful vaccine therefore needs to include the specific antigens for lymphocytes to recognise, and must also provide the secondary signal necessary for activation, differentiation and proliferation of the lymphocytes.

1.4.1.2 Exogenous Antigen Processing and CD4$^+$ T Cell Activation

Although APCs can process antigen through the endogenous pathway and present peptides in the context of MHC class I, as described above, internalised or exogenous proteins are present in a separate cellular compartment and are processed and presented by a distinct pathway (with some exceptions; see Cross Presentation below). They are bound to MHC class II molecules, unique to professional APCs, and are presented to CD4$^+$ T helper cells.

Following internalisation, exogenous antigen is processed in vesicles that become increasingly acidic with time. As the pH lowers, proteases within the endosome are activated, breaking down proteins into peptides (Brodsky and Guagliardi 1991; Watts 2004).

MHC class II molecules are present in the ER, but are prevented from binding protein from the cytosol that has been taken up by TAP (as MHC class I molecules do), by an associated molecule; the invariant chain. This molecule physically blocks the peptide-binding site of MHC class II and targets it to migrate to endosomes. When the invariant chain-MHC class II complex reaches a late-stage endosome, limited proteolysis of the invariant chain occurs, leaving a smaller peptide called CLIP. This molecule is then exchanged for antigenic peptides. The

MHC class II-peptide complex then migrates to the cell surface where the antigen is presented to $CD4^+$ T cell surveillance (Brodsky and Guagliardi 1991; Pieters 1997). This process is illustrated in Figures 1-4 (Processing of exogenous antigen) and 1-5 (MHC class II presentation).

Activation of $CD4^+$ cells is a similar process to the activation of $CD8^+$ cells described above. The TCR specifically recognises the peptide in the context of the MHC class II molecule and $CD4^+$ binds to invariant regions of MHC class II. Co-stimulatory signals are also required to activate naïve $CD4^+$ cells; these are the same as the secondary signals involved in $CD8^+$ activation.

It has been suggested that antigen is initially presented to naïve $CD4^+$ T cells exclusively by DCs within the T cell areas of secondary lymphoid tissues (lymph nodes, spleen and the Peyer's Patches in the intestinal wall) (Jenkins *et al.* 2001; Bajenoff and Guerder 2003). Other potential APCs such as macrophages and B cells are not found in the T cell areas of this tissue, and free antigen does not easily enter the LN. However, B cells are able to interact with T cells at the interface of the B and T cell compartments of the lymphoid tissues following encounter with specifically recognised antigen, and may be important in presenting blood-borne antigen to $CD4^+$ T cells (Baumgarth 2000; Mills and Cambier 2003). DCs are thought to acquire antigen by pinocytosis at the site where it entered the body or was expressed, rather than in the lymphoid tissue itself. Danger signals such as PAMP recognition and inflammation enhance $CD4^+$ T cell activation by stimulating DCs to migrate to T cell areas and upregulate their expression of MHC and co-stimulatory molecules.

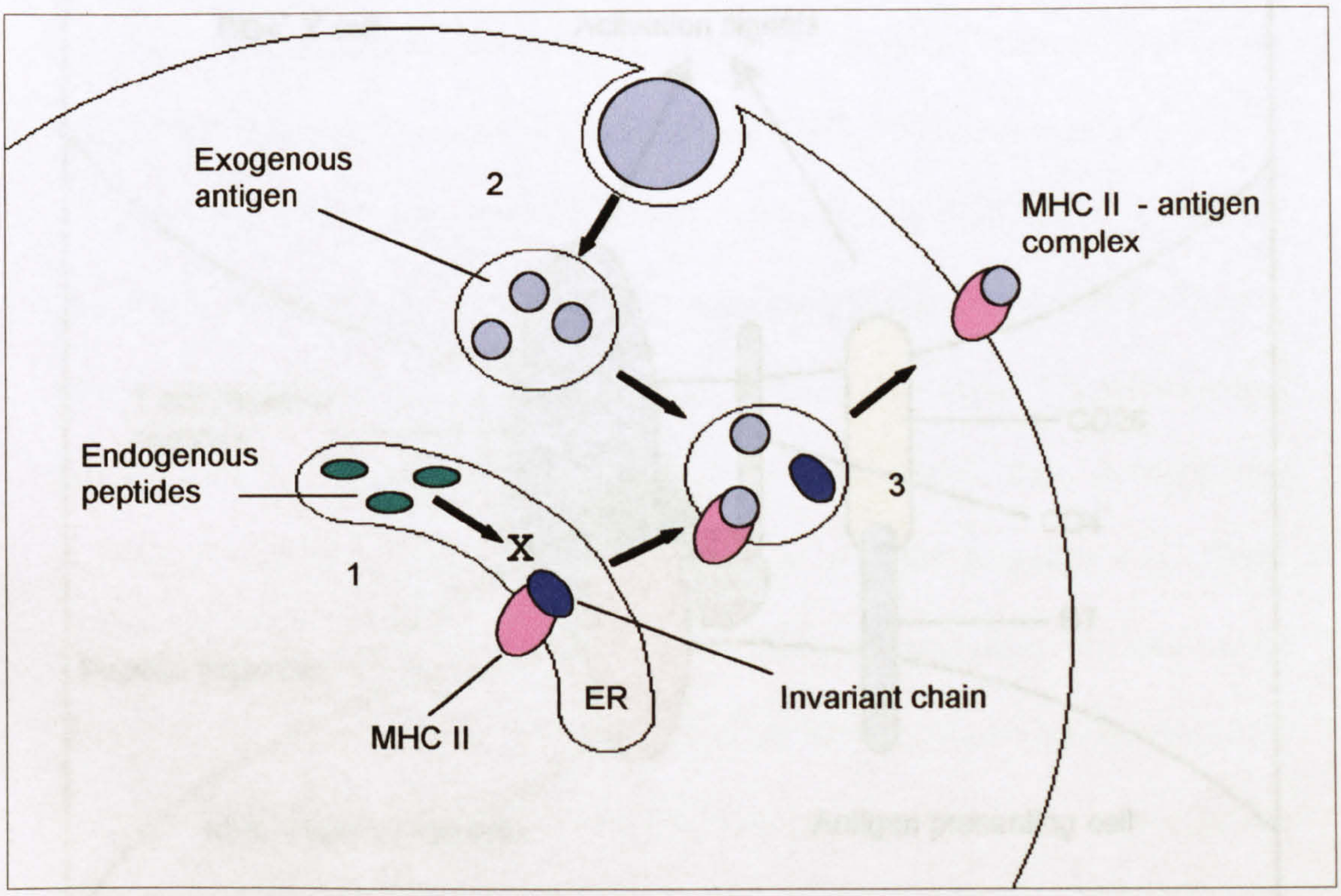


Figure 1-4: Processing of exogenous antigen

Although MHC class II molecules are present in the ER, they cannot bind to peptides in the ER due to the presence of the invariant chain (1). Extracellular material (exogenous antigen) is internalised and degraded by proteases and the progressively acidic conditions in endosomes (2). MHC class II molecules migrate to endosomes, where the invariant chain is partially cleaved by proteases. This allows binding of the degraded exogenous antigen. The MHC II – peptide complex is transported to the cell surface (3).

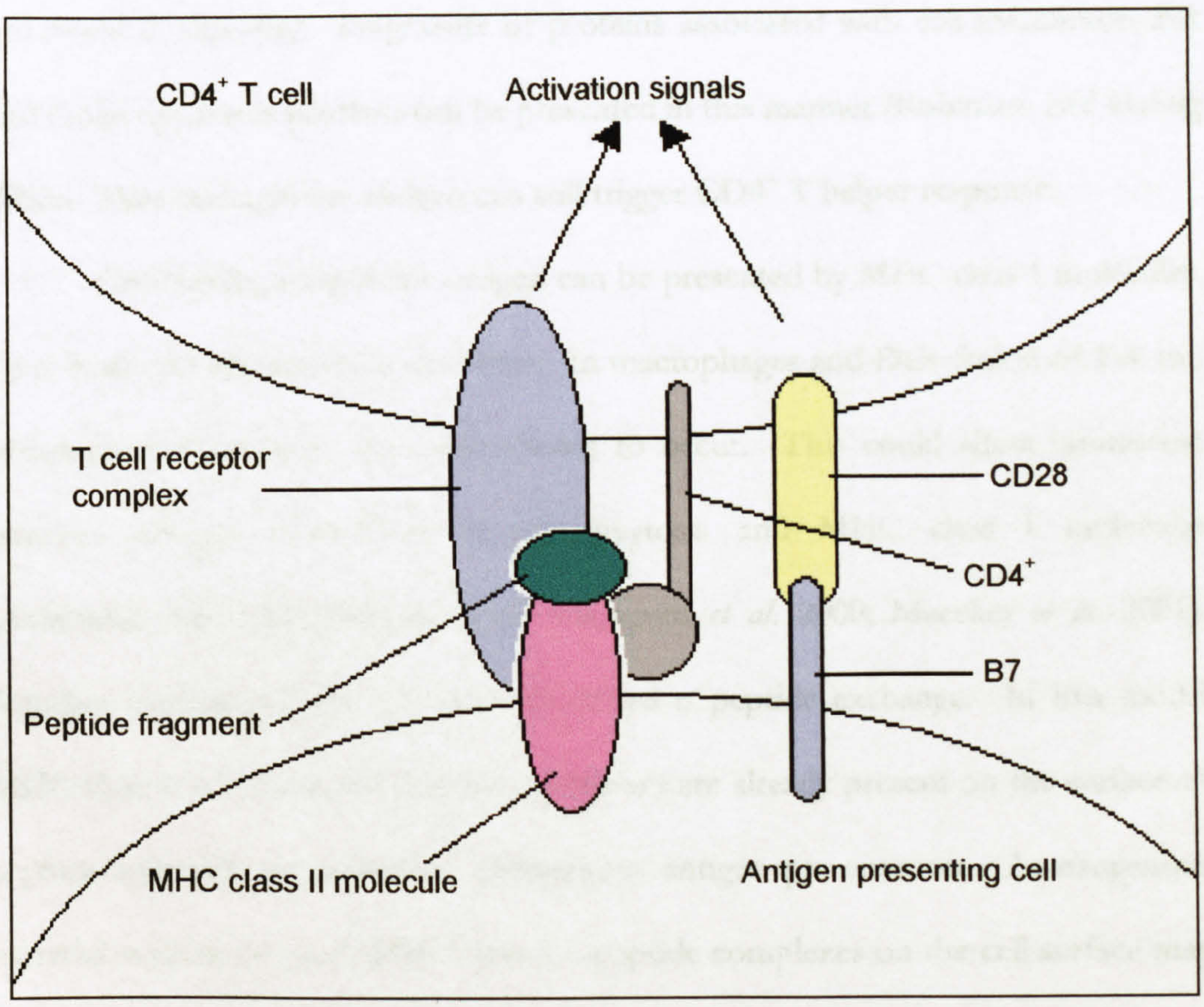


Figure 1-5: $CD4^+$ T cell antigen recognition and activation

This process is similar in many ways to the presentation of antigen and activation of $CD8^+$ T cells, the main differences being the peptide is presented by MHC class II molecules and the TCR is associated with $CD4^+$ on the T cell.

1.4.1.3 Cross Presentation – Crossover Between the Endogenous and Exogenous Pathways

It is worth noting that there are exceptions to the two main processing pathways outlined above: endogenous antigen can be presented in the context of the MHC class II molecule and exogenous antigen in that of MHC class I.

Although molecules found in the cytosol are rarely processed and presented in complex with MHC class II in professional APCs, other cellular proteins can be processed in this way. Fragments of proteins associated with cell membrane, ER and Golgi apparatus proteins can be presented in this manner (Robinson and Delvig 2002). Thus endogenous antigen can still trigger $CD4^+$ T helper responses.

Conversely, exogenous antigen can be presented by MHC class I molecules, by at least one of several mechanisms. In macrophages and DCs fusion of ER and phagosome membranes has been shown to occur. This could allow interaction between antigen internalised by phagocytosis and MHC class I molecules (Ackerman and Cresswell 2004); (Kumaraguru *et al.* 2000; Maecker *et al.* 2001). Another mechanism that has been described is peptide exchange. In this model MHC class I – endogenous peptide complexes are already present on the surface of a phagocytic cell, as in normal endogenous antigen presentation. As exogenous material is phagocytosed, MHC class I – peptide complexes on the cell surface may be simultaneously internalised. The conditions in the lysosome degrade the exogenous antigen to peptides able to bind to MHC class I molecule, which can then be exchanged for the already bound endogenous peptides. This second mechanism is considered as being less likely to occur *in vivo*, since the proteolytic environment of the endocytic pathway is unlikely to reproducibly generate the same peptides as produced by the proteasomes that normally produce MHC class I epitopes (Lehner and Cresswell 2004).

While the mechanism(s) behind cross-priming are not fully understood, it is a phenomenon that has been observed repeatedly. One example particularly relevant to this project is the processing of particulate exogenous antigen by DCs and subsequent efficient presentation to $CD8^+$ T cells (Stober *et al.* 2002). It is therefore possible for exogenous antigen to stimulate activation of CTLs.

1.4.1.4 B Lymphocyte Antigen Recognition and Activation

B cell specificity is defined by a process of somatic recombination (similar to that of T cells) within the genes of immunoglobulin, which code for the B cell receptor (BCR). This occurs during development of the lymphocyte in the bone marrow, and is limited only to exclude specificities for self-antigens. Previously activated memory B cells can be triggered by antigen specific recognition alone (Hebeis *et al.* 2004), in the same way as memory T lymphocytes.

However, recognition of antigen alone is usually insufficient for activation of naïve B cells. As with T cells, a secondary signal is required, often in the form of antigen-specific interactions between an activated Th cell and the naïve B cell which is presenting the antigen. This standard model of B lymphocyte activation was first proposed over 30 years ago (Bretscher and Cohn 1970). According to the current version of this model, B cells internalise and process antigen through the BCR, providing the first activation signal necessary. However, as with T lymphocytes, signal one is not sufficient to initiate clonal expansion and differentiation of naïve B cells.

Segregation of B and Th cells in secondary lymph nodes is maintained by chemokine gradients to which the naïve lymphocytes respond differentially. Within hours of encountering antigen, B cells increase chemokine receptor CCR7 expression, enhancing responsiveness to T cell chemokines. Likewise, activated T

cells express receptors for follicular zone chemokines. Thus B cells that have encountered antigen and activated T cells are able to migrate to the boundaries of cortical T and follicular B cell zones (Mills and Cambier 2003). When these cells come into contact, then antigen specific TCR/MHC-peptide interaction can induce the expression of secondary signal molecules in the B cell. These include CD40 ligand and IL-4, -5 and -6, initiating proliferation of the B cell and differentiation into an antibody secreting plasma cell (Baumgarth 2000; Mills and Cambier 2003).

However, T cell-independent B cell activation mechanisms also exist. These require T cell-independent antigens, or antigens plus secondary signals from a source other than Th cells, and can induce a full B cell response. These alternative means of activation potentially provide a more rapid humoral response, since they can be initiated without the prior clonal amplification of Th cells, a process that takes several days (Baumgarth 2000). Some multivalent, polymeric antigens, such as the hepatitis B core antigen (see below), are able to simultaneously bind simultaneously to several BCR molecules. This extensive cross-linking of the receptor molecules can be sufficient to initiate activation of the B cell without T cell help (Milich and McLachlan 1986). B cell mitogens, for example LPS, can also activate B cells regardless of their antigen specificity.

1.4.2 T Cell Effector Functions

All T cell functions are dependent on interaction between an activated T cell and a target antigen-specific presenting cell. The functions performed by T cells depend upon the array of effector molecules that they secrete in response to being triggered. These include tightly regulated cytotoxins, such as those released by CTLs to kill virally infected cells, and cytokines that can act locally or at a distance

by binding to cell receptors. This results in a change in the behaviour or properties of the target cell.

1.4.2.1 CD8⁺ T Cell Effector Responses

While humoral responses, supported by $CD4^+$ T cell help, provide protection in the extracellular spaces in the body, activated $CD8^+$ T cells, or CTLs, combat intracellular pathogens by effector cytokine secretion and killing of cells presenting foreign antigens.

An activated CTL that encounters a recognised peptide-MHC class I molecule will be triggered to kill the presenting cell. The principal way in which this occurs is through the calcium dependent release of specialised lysosomes following recognition. These contain perforin, a molecule that enters the membrane of the target cell and polymerises to form a transmembrane pore. The presence of this pore disrupts the integrity of the membrane and allows free flow of water and salts in and out of the cell. In addition, proteases known as granzymes are released from the same lysosomes as perforin. On formation of the transmembrane pore, the granzymes are able to enter the target cell. Granzymes activate caspase enzymes, triggering a chain of events that ends with degradation of DNA and the induction of apoptosis (Barry and Bleackley 2002). CTLs also express Fas ligand. When the T cell and target cell bound together in an antigen-specific reaction, Fas ligand can bind to Fas receptors ('death receptors') in the target cell membrane. Ligation of Fas also activates caspase molecules. Fas activated apoptosis is believed to be an important mechanism in regulating peripheral immune responses; once antigen is no longer present, the majority of antigen-specific lymphocytes are killed via this mechanism (Seki *et al.* 2002; Dockrell 2003).

Apoptosis is an induced form of cell death, regulated by carefully controlled cellular pathways. Unlike necrosis, it results in the release of fragments of cellular material in vesicles or 'blebs', which are taken up by phagocytic cells. Chromosomal and cytosolic DNA are degraded into small fragments, and proteases are activated. These mechanisms ensure the destruction of intracellular pathogens and uptake of dying cell by macrophages but importantly does not encourage an inflammatory response. In contrast, necrotic cell death allows the escape of intracellular pathogens and the release of molecules that induce an inflammatory response. The two forms of cell death therefore have very different immunological outcomes, and apoptosis results in significantly less immunopathology than necrosis.

As well as directly killing cells, activated CTLs secrete cytokines such as tumour necrosis factors (TNF) -α and -β and IFN-γ. These molecules act non-specifically and do not cause cell death. Instead they contribute to host defence in various different ways. TNFs and IFNs upregulate Natural Killer cell activity and IFN-γ directly inhibits viral replication and increases expression of proteins involved in the expression of antigen, such as MHC class I, in infected cells. IFN-γ also activates macrophages in conjunction with another signal such as the presence of LPS. It reduces intracellular tryptophan concentrations, which can inhibit the growth of intracellular pathogens by effectively starving them, and increases the efficacy of Fas-and perforin-mediated cytotoxicity by CTLs (Roth and Pircher 2004). TNF-α and TNF-β also activate macrophages, acting synergistically with IFN-γ, and can induce killing in some target cells through TNF receptor I.

1.4.2.2 CD4⁺ T Cell Responses

$CD4^+$ Th cells aid in the activation, enhance the activity and encourage proliferation of other immune cells. They can activate macrophages to kill intracellular bacteria, induce proliferation and class switching in B cells and help activate naïve $CD8^+$ T cells (see above). Like $CD8^+$ T cells, their effects are mediated by direct cellular interactions and cytokine secretion following antigen specific recognition.

Physical detection of antigen-specific $CD4^+$ T cells has revealed features of the *in vivo* immune response that were not appreciated from previous *in vitro* studies. *In vivo*, antigen is initially presented to naive $CD4^+$ T cells mainly by DCs within the T cell areas of secondary lymphoid tissues. Anatomical constraints make it likely that these DCs acquire the antigen at the site where it enters the body. Inflammation enhances T cell activation by stimulating DCs to migrate to the T cell areas and upregulate MHC and co-stimulatory molecule expression. Inflammatory signals induce chemokine receptors on activated T cells that direct their migration toward B cell follicles to interact with antigen-specific B cells (Jenkins *et al.* 2001).

T helper cells have been shown to be important in the development of a strong memory response. Proliferation of memory $CD8^+$ T lymphocytes and the level of cytokine production they display are dependent on the presence of $CD4^+$ T cells during an initial recognition of antigen (Shedlock and Shen 2003; Sun and Bevan 2003).

The cytokine environment generated at the time of priming is also important in the differentiation of the T cell. This can come from previously activated helper cells or from the APC itself and directs the T helper bias of the ensuing clonal population. $CD4^+$ Th cells fall into two main categories; Th1 cells and Th2 cells, depending on the pattern of cytokines they generate following

activation. Broadly speaking, Th1 cells promote inflammatory responses and activate macrophages by the production of IL-2, IFN-γ, IL-6 and IL-12, and Th2 cells support the expansion of activated B cells through the generation of IL-4, IL-5 and IL-10 (Mosmann and Sad 1996).

Successful protection against some diseases, such as tuberculosis, can be significantly affected by the expansion of particular subsets of Th cells (Tanghe, D'Souza et al 2001).

Th1 $CD4^+$ Responses

Th1 cells co-ordinate the immune response against intracellular microorganisms. Central to this is the antigen-specific activation of macrophages. Some bacteria such as *Salmonella* and mycobacteria are able to survive and grow in the phagolysosomes of macrophages after phagocytosis. In this environment they are protected from CTLs and antibodies. They survive by specifically inhibiting the normal killing process of acidification of the phagosome, proteolysis and fusion with lysosomes used by macrophages. However, activation of macrophage by a Th1 cell following antigen recognition can eliminate such intracellular pathogens by enhancing the killing mechanisms within the cell. Two signals are required for macrophage activation: IFN-γ and one of several other potential signals, such as CD40L, TNF-α or TNF-β binding. These signals can be simultaneously be provided by an activated $CD4^+$ Th1 cell secreting appropriate inflammatory cytokines.

The Th1 cytokine IFN-γ also induces B cells to switch the class of immunoglobulin subtype they produce, usually in mice from IgM to IgG2a. This

subtype of antibodies has been correlated with strong opsonisation activity (see section 1.4.3 Humoral Immune Responses).

Th2 CD4$^+$ Responses

Th2 cells on the other hand activate naïve B cells to proliferate and produce IgM. The cytokines they secrete also drive class-switching to production and secretion of other types of antibody, including IgG1, IgA and IgE. These antibodies are more important in the control of extracellular pathogens, and are efficient at neutralisation and opsonisation of pathogens. Also IgA in its secretory form has the capacity to translocate the mucosal epithelium, neutralising microbes at the mucosal surfaces. Details of the effector functions of antibodies are given below (section 1.4.3)

Factors Affecting the Development of a Th1 or Th2 Outcome

The differentiation of a naïve CD4$^+$ T into a Th1 or Th2 type of cell appears to be heavily dependent upon the microenvironment surrounding the cell when it is activated. There are several factors that appear to influence this environment, including the local production of cytokines. These can have a major influence on Th1/Th2 outcome and their presence is largely dependent on the type of cells that are present and activated. Macrophages and NK cells secrete pro-Th1 cytokines such as TNF-α and IL-12. Mast cells and B cells secrete the pro-Th2 cytokines IL-4 and IL-10. As well as the cytokines present, the concentration of activating antigen can affect the outcome, with higher concentration of antigen resulting in a tendency toward Th1 cell development. In addition, in mice there appears to be a balance between these types of responses. IFN-γ inhibits Th2 cell growth and IL-10 inhibits Th1 cell growth. This action can lead to strong polarisation of response.

1.4.3 Humoral Immune Responses

B cells develop in the bone marrow and in their naïve state traffic through the B cell follicles of secondary lymphoid tissues. On activation, they proliferate in germinal centres, begin to secrete antibody and undergo somatic hypermutation (to generate increased diversity of antibody specificity). During this stage of differentiation, the B cell population undergoes affinity maturation; cells compete to bind antigen and those with poor affinity are selected out. Class switching of antibody production can be influenced by cytokines secreted by T helper cells, for example, in order to produce antibody with different effector functions.

Antibodies protect extracellular spaces within the body and mucosal surfaces at the interface between the body and the external environment. They function by binding to molecules or microbes in an antigen-specific manner. They are structurally similar to the BCR and their specificity is determined by the same somatically recombined genes, therefore the antibody produced by a plasma cell will bind to the same antigen that was initially bound by the BCR. Figure 1-6 shows a schematic diagram of an antibody showing the variable antigen-binding and the constant regions.

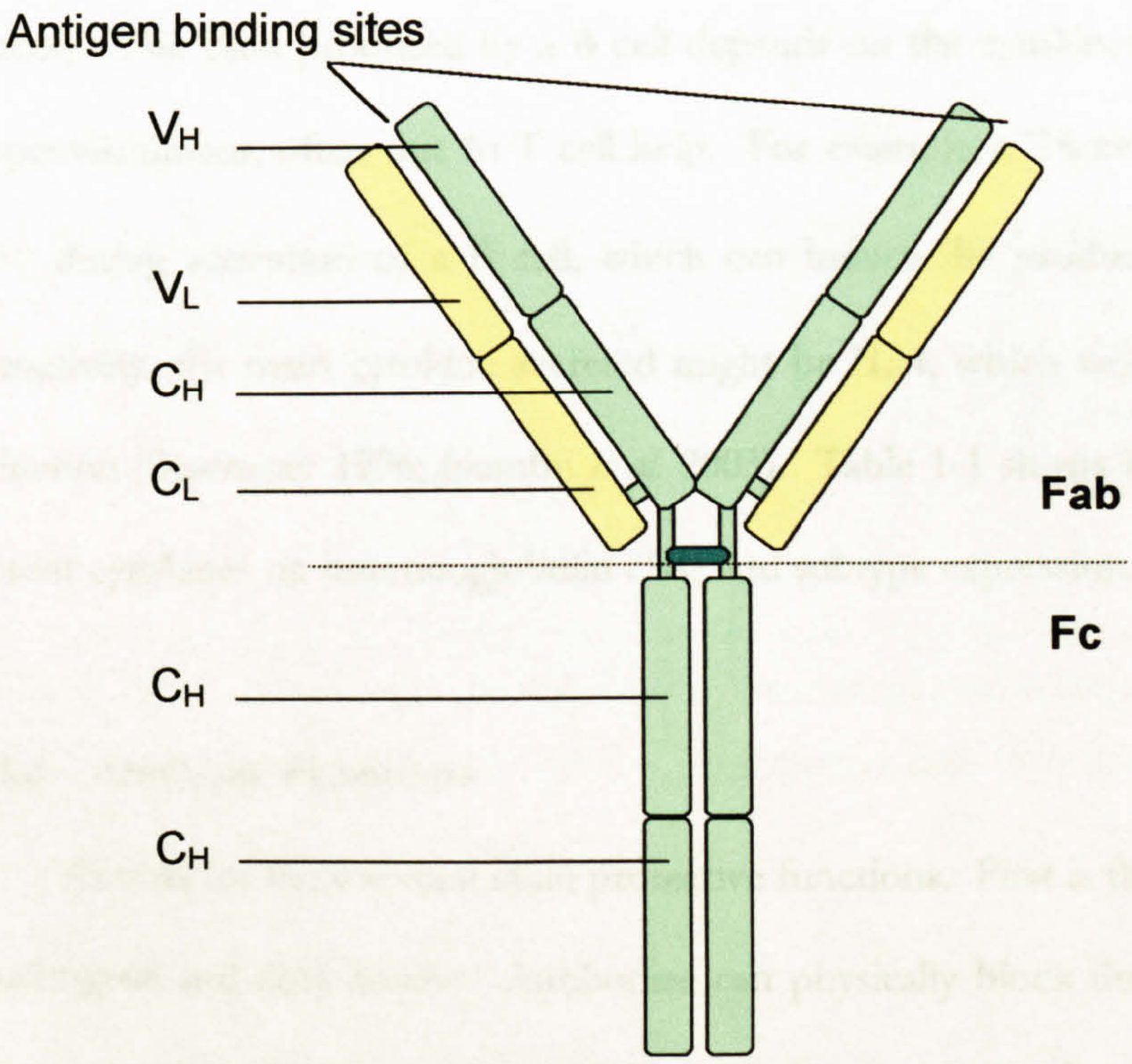


Figure 1-6: Schematic IgG antibody molecule

This diagram shows the functional and structural domains of immunoglobulin G. The molecule is made up of light ($_L$) and heavy ($_H$) chains with variable (V) and constant (C) domains. The molecule is bivalent, with a pair of light and heavy variable domains making up each antigen binding site. The entire antibody can be divided into two fragments: an antigen-binding fragment (Fab) and a crystallisable fragment (Fc) the latter of which can bind to Fc receptors on the surface of B cells and other APCs.

1.4.3.1 Antibody Classes and Subtypes

Different classes of antibody are produced by different plasma cells and each has the capacity to induce the mechanisms outlined above to a different degree (Corbeil 2002).

The different classes of antibody are determined by the gene from which the heavy chain is transcribed. The different classes of antibody include Immunoglobulin (Ig) M and IgD (expressed by naïve B cells), IgG (the main class found in serum during infection) and IgA (a form of which can cross mucosal surfaces). The class produced by a B cell depends on the cytokines present in the microenvironment, often due to T cell help. For example, a Th cell might secrete IFN-γ during activation of a B cell, which can induce the production of IgG2a. Alternatively, the main cytokine secreted might be IL-4, which would cause IgG1 production (Stavnezer 1996; Nambu *et al.* 2003). Table 1-1 shows the influence of different cytokines on immunoglobulin class and subtype expression.

1.4.3.2 Antibody Functions

Antibodies have several main protective functions. First is the neutralisation of pathogens and their toxins. Antibodies can physically block the active sites of toxin molecules, for example, inhibiting their enzymatic function or attach themselves to ligands on the bacterial surface, prevent them from binding to receptors on host cells. Any functionally important molecule that is exposed to antibody binding can thus be a target of neutralisation.

	IgM	IgG1	IgG2a	IgG2b	IgA
IL-4	Inhibits	Induces	Inhibits	-	-
IL-5	-	-	-	-	Augments
IFN-γ	Inhibits	Inhibits	Induces	-	-
TGF-β	Inhibits	-	-	Induces	Induces

Table 1-1: Role of cytokines in regulating immunoglobulin expression

Adapted from (Janeway *et al.* 2001), (Tokuyama and Tokuyama 1999) and (Michaelsen *et al.* 2004).

The second function is opsonisation; the promotion of phagocytosis. Certain immune effector cells such as macrophages are able to bind to antibodies via the Fc region (see Figure 1-6) once the antibody is bound to its target, facilitating phagocytosis.

Antibodies can trigger the complement cascade, which is itself able to enhance opsonisation and induce lysis of some bacteria (see section 1.3, Innate Immunity;(Ehrenstein *et al.* 1998).

Antibodies can also bind to viral proteins expressed on the surface of infected self-cells and allow the binding of NK cells that can kill the infected cell. This process is known as antibody-dependent cell-mediated cytotoxicity (Moretta *et al.* 2003).

The constant region of the immunoglobulin molecule determines its antibody class and influences the functional activity of the molecule. The Fc region of different immunoglobulin classes are recognised by Fc receptors on the surfaces of different immune effector cells, allowing each antibody class to interact with distinct subsets of immune cells. For example IgG1 Fc is recognised by macrophage and neutrophil receptors, enhancing the phagocytosis of pathogens coated with this immunoglobulin subtype (Barrington *et al.* 2001).

The Fc portion of an antibody-antigen complex can bind to the C1q molecule of complement, enhancing the uptake of immune complexes by phagocytes and activating the complement cascade (Brekke *et al.* 1995).

IgM can strongly activate complement, thus providing an alternative protective mechanism early in infection, when high levels of IgM are present but specific cellular responses have not reached their maximum. IgM is also believed to have a role in the development of a strong IgG response (Ehrenstein *et al.* 1998).

The Fc region enables the active transport of antibody to areas where it would not otherwise be present, for example the mucosal surfaces. This is accomplished by binding of the Fc to receptors allowing the translocation of the molecule through epithelial barriers. This is an important protective measure, bringing the protective mechanisms of antibodies to these areas. Most types of antibody cannot cross the epithelia, but secretory IgA (sIgA) is able to do so, as is IgM to a lesser extent. SIgA is produced by plasma cells residing in the lamina propria, adjacent to the basal membrane of the epithelial surfaces. The sIgA molecule is a dimer consisting of two individual IgA molecules joined by a J chain. Poly-immunoglobulin receptors on the basal surface of epithelial cells bind sIgA. Binding is followed by internalisation, active transport across the cell and release into e.g. the bronchial or intestinal lumen (Rojas and Apodaca 2002). This form of IgA is able to neutralise pathogens and toxins at the epithelial surfaces. Thus sIgA provides antigen-specific defence at the mucosal surfaces in addition to innate mechanisms.

In contrast, IgG acts to protect the extracellular spaces of the internal tissues. It has a relatively long half-life and like IgA can neutralise pathogens and toxins. In addition it can activate complement and opsonise pathogens. It is the main class of immunoglobulin found in the serum during infection. IgG2a is effective at opsonising material for phagocytosis and activating complement (Clark 1997; Michaelsen *et al.* 2004). The main functions of IgM are efficient neutralisation (due to its pentameric structure) and activation of complement.

1.4.4 Lymphocyte Migration

Lymphocyte action and immune responses as a whole can be restricted to certain body compartments. This provides an immune response where necessary, enabling the efficient clearance of pathogens whilst limiting the potential for widespread immunopathogenesis. The tropism of lymphocytes is regulated by the expression of surface adhesion molecules, such as integrins. Naïve lymphocytes display a lymph node-specific tropism, causing them to reside in areas where they are likely to encounter APCs. Expression of different adhesion molecules might, for example, direct activated cells to migrate to a site of inflammation (Sallusto *et al.* 1999). A consequence of this is that different routes of vaccination may or may not synergise to provide stronger overall response, depending on which immunological compartment the individual responses occur in, and the flow of antigen, APCs and lymphocytes between the compartments.

1.4.5 Generation of Memory T and B Lymphocytes

Immunologic memory gives the immune system the ability to respond more rapidly and effectively to pathogens that have been previously encountered. Activated lymphocytes develop either into the effector cells that provide the functions described above, or into memory cells. Whilst effector cells are responsible for conferring immediate defence against pathogens, memory cells are important for providing immunity against re-infection, or in the case of a response stimulated by vaccination, long-term defence against a natural infection.

When an infection has been cleared, or vaccine antigen is removed by the immune system, the ongoing signals that stimulate an effector response are removed. In the absence of these signals, the majority of effector cells die through

apoptosis (Masopust *et al.* 2001). Memory T and B cells are a distinct subset of the clonally expanded lymphocyte population that has undergone affinity selection for the antigens presented during immunisation. Unlike effector cells, they can persist without residual antigen. The generation of such a population is believed to be proportionate to the number of effector cells activated, thus measurement of strong immune responses based on the initial effector population is predictive of the development of strong secondary responses on subsequent challenge. Th cells are important in the development of the memory response. Proliferation of memory lymphocytes is dependent on the presence of $CD4^+$ T cells during an initial vaccination (Sun and Bevan 2003).

A secondary or subsequent humoral response to a pathogen is characterised by a much higher level of IgG production. IgM is still present in the secondary response, at similar levels to those measured during the primary response. IgA and IgE are also present to a greater degree during memory responses. Individual antibody molecules exhibit a higher affinity for antigen, due to somatic hypermutation in the V regions of immunoglobulin genes and antigen-binding dependent selection in the lymph nodes. The affinity continues to increase during secondary and subsequent immune responses, therefore it can be advantageous to deliver multiple doses of a vaccine over a period of time. The increased affinity of surface antibody as well as greater expression of MHC class II molecules facilitate antigen uptake and presentation, giving a greater sensitivity to low antigen concentrations. Memory B cells persist in the germinal centres of the secondary lymphoid organs (Ochsenbein *et al.* 2000).

Memory T cells, on the other hand, can traffic to various tissues depending on which surface adhesion molecules are expressed, by which they can be distinguished from effector T cells. Like memory B cells, such T cells are long lived

and can be observed at elevated numbers following the resolution of an infection. There are two subsets of memory T cells: effector memory T cells and central memory T cells. The effector cells circulate in the periphery and can be activated much more rapidly than central memory cells, which persist in the lymphoid tissues and may possess greater longevity. The two populations of memory cells are thought to provide two different functions in the event of re-exposure to antigen. The peripheral cells are able to provide a rapid effector response whilst the central cells promote secondary proliferative responses (Masopust *et al.* 2001; Sallusto *et al.* 2004).

1.5 Types of Vaccine

The goal of a vaccine is to stimulate the immune cells described above to confer protection against a particular pathogen and to maintain this protection over a long period of time. Outlined below are some conventional types of vaccines including those examined in this thesis.

1.5.1 Killed Whole-Cell Vaccines

Killed whole cell vaccines are composed of entire pathogen preparations, whose viability has been destroyed by chemical and/or heat treatment. These types of vaccine are considered safer than live, attenuated vaccines as there is no danger of reversion to a virulent phenotype. However, they are generally less immunogenic and often require multiple doses to be effective. Examples of chemically inactivated vaccines are those protecting against rabies virus, *Bordetella pertussis* (the causative agent of whooping cough, although an acellular vaccine has recently been

introduced in the UK), and the Salk polio vaccine. This vaccine, in which wild-type virus is inactivated by treatment with formalin, is more expensive to produce than the attenuated vaccine, and is less immunogenic, but its improved safety may render it a more appropriate choice in regions were there are few natural cases of polio (Imam 1981). Despite the high efficacy of killed vaccines, side effects are not uncommon, and such vaccines are used in humans only when safer alternatives are not available. In animals, however, safety concerns are often less stringent, and whole-cell killed vaccines are in common use (Cobo *et al.* 2002; Armijos *et al.* 2004).

1.5.2 Subunit Vaccines

Acellular or subunit vaccines consist of the purified components of pathogens. They are believed to be safer than either killed whole-organism or live, attenuated vaccines, since a vaccine based on a whole organism can contain toxic components, even if the microbe itself is non-viable. Subunit vaccines contain a few or even a single purified antigen from the original pathogen. Recombinant subunit vaccines are precisely defined and are usually safe and well tolerated. Examples of successful subunit vaccines include Hepatitis B surface antigen and the acellular *Bordetella pertussis* vaccine. The drawback to producing such vaccines is that many purified antigens do not elicit a strong immune response. This can be because too few antigens are included in the formulation or the removal of inflammatory molecules and PAMPs from the whole cell formulation reduces the potential to activate the immune system. Without these triggers, many antigens are not immunogenic and must be administered with an adjuvant – a substance that increases the immune response to co-administered molecules. To date the type of adjuvants that have been licensed for use in humans remain limited to aluminium

salts (alum), which has been in use for decades, or, more recently, detoxified bacterial molecules (e.g. monophosphoryl lipid A (Baldrick *et al.* 2002)). A consequence of using alum is that subsequent immunity tends to be biased strongly towards a Th2 response; high antibody production but little cell-mediated immunity, whereas bacterial derivatives can induce Th1 responses (Lodmell *et al.* 2000).

1.5.3 Live-Attenuated Vaccines

Live, attenuated vaccines are generally powerful vaccines, reproducing many of the features of a natural infection including some degree of replication and survival of the organism in the body. This characteristic makes attenuated vaccines potentially more likely to generate protective immunity than killed organisms or subunit vaccines. However, attenuated vaccine strains can potentially revert to a virulent state resulting in disease. This has been observed with the Sabin oral polio vaccine, where reversion to virulence occurs in about one vaccinee per million and can result in poliomyelitis (Wood and Thorley 2003). Therefore the utility of this vaccine depends on the conditions in which it is employed. As a cheap, easily administered and highly effective vaccine, for treatment in endemic regions, this type of vaccine is invaluable. However, in a population from which the virus has been largely eliminated, the use of this vaccine can be the cause of the majority of cases of poliomyelitis (Plotkin 2003; Yang *et al.* 2003). In these circumstances it may become appropriate to use an inactivated vaccine that is less immunogenic but shows a greater safety profile (Wood and Thorley 2003).

1.5.3.1 Mucosal Vaccination and Immunology

The single greatest advantage of mucosal vaccination is its potential to invoke immunity at the mucosal surface as well as systemically. Since the majority of pathogens invade the body through the mucosal surfaces, the mucosal immune response can prevent the initial entry of the organism into the host and thus prevent systemic disease. This effectively provides an earlier line of defence against infection and can additionally protect against infectious organisms that cause damage to these surfaces by the production of toxins. Protection is partially mediated by secretory IgA, which is not induced by parenteral vaccination (Johnson *et al.* 1995). Similarly, systemic vaccination fails to induce cell-mediated immunity at mucosal membranes, whilst delivery of a vaccine directly to the mucosal surfaces can induce such responses (Gallichan and Rosenthal 1996; Kanellos *et al.* 2000). In addition, it is believed that trafficking of lymphocytes between mucosal surfaces is linked and that a vaccine delivered to one part of the mucosal immune system can induce immunity at some distal mucosal sites. For example, oral immunisation can confer an immune response in the upper respiratory tract as well as in the intestinal mucosa. This is generally known as the common mucosal immune response and makes mucosal immunisation particularly attractive (Chen 2000).

Mucosal immunisation also offers practical advantages for vaccine delivery. Since there is no hypodermic administration of the vaccine, blood-borne diseases such as HIV and hepatitis cannot be passed from one recipient to another by re-use of contaminated needles. Nor are medical staff at risk of infection due to accidental needlestick injuries, currently a frequent occurrence (Elmiyeh *et al.* 2004). Painless, simple and non-invasive, mucosal administration can also lead to increased uptake in a population.

A potential problem with mucosal vaccination is the difficulty of generating mucosal immune responses. The normal response to mucosal antigens is anergy or tolerance, since an inappropriate response against innocuous airborne molecules or proteins in food, for example, could be harmful (Dougan *et al.* 2000). Secondary signals must be provided for the activation of naïve lymphocytes. These signals can be triggered by bacterial toxins such as *E. coli* LT or Cholera toxin (Rappuoli *et al.* 1999), which can be detoxified and yet remain effective as mucosal adjuvants (Pizza *et al.* 2001).

1.5.3.2 Use of Attenuated Vectors

Despite the advantages outlined above, there are few mucosal vaccines in use. This is mainly because of the difficulty of generating mucosal responses. However, live organisms, even though attenuated, can overcome mucosal tolerance through the activation of innate immunity (Dougan *et al.* 2000; Huang *et al.* 2001; Woo *et al.* 2001).

The ability to replicate, even at a reduced rate, confers increased longevity of antigen expression and stimulation of the immune system (compared to a non-living vaccination system). Also, immunisation with attenuated vectors can generate immune responses to the vector, which can induce protective responses against related pathogens, as has been shown with *Salmonella* (Salerno-Goncalves *et al.* 2003), as well as to any heterologous antigens that are expressed by this organism. This can be achieved by genetic manipulation of the attenuated vaccine to allow expression of a heterologous antigen (Medina and Guzman 2001). Therefore this strategy has the potential to simultaneously vaccinate against more than one pathogen.

One problem associated with the use of attenuated vector vaccines is pre-existing immunity to the vaccine organism in geographical regions where the wild-type organism is endemic. This might result in early clearance of the vector and reduced exposure to the vaccine antigens, giving dramatically reduced immune responses (Medina and Guzman 2001). To overcome this, higher doses of bacteria can be used, but not without introducing the danger of giving too high a dose to non-immune individuals. Conversely, the calculated dose might be too high in non-endemic areas if vaccine is originally tested in endemic infection area (Attridge *et al.* 1997). However, this theoretical risk may not be important, since it has been shown that prior immunologic experience of a *Salmonella* vaccine carrier strain (or similar strains of *Salmonella*) can potentiate a stronger humoral immune response than is seen without prior exposure (Bao and Clements 1991).

A wide range of different bacteria and attenuation mutants are being evaluated for vaccine delivery, but *Salmonella* is one of the most studied genera, with several examples of human clinical trials (Medina and Guzman 2001). Such studies have considered their use in human typhoid fever vaccines (Salerno-Goncalves *et al.* 2003), for use as carriers to deliver DNA vaccines to host cells (Woo *et al.* 2001), or to deliver vaccine antigens of other pathogens in humans (Bumann *et al.* 2000) and mice (Foynes *et al.* 2003).

1.5.3.3 Use of Salmonella typhimurium as a live attenuated delivery strain

Salmonella enterica, serovar *typhimurium*, is referred to as *S. typhimurium* throughout this thesis. It is a Gram negative bacterium which when used to infect mice causes a systemic disease with many of the features associated with human typhoid fever.

Salmonella can enter the body through gut epithelial cells or M cells, specialist antigen sampling cells on the surface of the Peyer's patches in the intestinal epithelium. From here they are able to invade macrophages and DCs and can survive in the phagolysosomes of these cells. This is despite the intracellular killing mechanisms that exist within macrophages to eliminate intracellular pathogens (Carrol *et al.* 1979). Migration of such infected cells can lead to systemic infection, with dissemination to the spleen, liver and lymph nodes. The presence of the bacteria at different sites enables the activation of responses both systemically and at mucosal surfaces (Medina and Guzman 2000).

The presence of highly immunogenic components of the bacteria including LPS and flagellin and the invasion of immune cells such macrophages activate mechanisms which can enhance specific immune responses. This is mediated through the release of pro-inflammatory cytokines, such as IL-1 and TNF-α, resulting in an influx of neutrophils, DCs and T lymphocytes to regions with bacteria-carrying cells and upregulating APC co-stimulatory signals via TLRs (Takeda and Akira 2001).

Control of infection is mainly by acquired T cell responses: including enhancement of macrophage killing mechanisms by Th1 cells and direct killing of infected cells by CTLs (Mastroeni and Sheppard 2004). This occurs more rapidly with attenuated vaccine strains, but does not prevent the presentation of antigens encoded by the bacteria, or the dissemination of the bacteria around the body.

1.5.3.4 Attenuation of S. typhimurium BRD 509

Live bacteria used to deliver vaccines must be attenuated to the extent that they cannot cause disease in the vaccine recipient. *Salmonella* is a much studied organism, and its virulence factors have been well characterised (Hensel *et al.* 1995;

Marcus *et al.* 2000). This has facilitated the creation of mutants lacking various virulence factors (O'Callaghan *et al.* 1988). One effective vaccine candidate is *S. typhimurium* BRD509. The strain is attenuated by deletion of two genes; *aroA* and *aroD*, encoding enzymes in the biosynthetic pathways involved in the generation of the aromatic amino acids (Strugnell *et al.* 1992). The bacteria are therefore auxotrophic, i.e. dependent on external supply of these amino acids. The *aro* genes were selected for mutation because aromatic metabolites are not usually present in human tissues, therefore mutants should be unable to replicate at these sites. Their diminished ability to replicate *in vivo* results in a self-limiting, sub-clinical infection, but they remain able to express and present vaccine antigen (Chatfield *et al.* 1993). Administration of this strain expressing antigens from other pathogens has been shown to result in mucosal responses offering partial protection against a mucosal pathogen (Strugnell *et al.* 1992). Systemic antibody production against the antigens of the secondary vaccine candidate has also been observed (Roberts *et al.* 2000), although T helper cell responses were limited to *Salmonella* antigens (Londono *et al.* 1996).

1.5.3.5 Antigen Expression

The production and presence of a heterologous antigen lays a considerable metabolic burden on recombinant bacteria. This can result in reduced growth and decreased plasmid stability. Attempts to reduce the impact of antigen expression on plasmid stability have included the use of inducible promoters as an alternative to constitutive antigen expression. One commonly described promoter is *nirB*, which is activated under anaerobic conditions such as the interior of eukaryotic cells (Bell *et al.* 1990). This allows growth *in vitro* where oxygenation of the culture is high, whilst allowing antigen expression on vaccination in the tissue when the oxygen

tension is low. There have been several comparisons between strains expressing antigens under the control of different promoters. Two groups have demonstrated greater plasmid stability both *in vitro* and *in vivo* with the use of *nirB* than equivalent plasmids from which an antigen is constitutively expressed (Oxer *et al.* 1991; Chatfield *et al.* 1992). The *nirB* promoter has also been shown to drive higher levels of expression in *S. typhimurium aroA* mutants than *pagC* under conditions in which both promoters were induced. In this case, plasmids expressing the same antigen under the control of these promoters were shown to have similar levels of stability *in vitro*. However, stability of both plasmids was reduced *in vivo*, although *nirB* was maintained at a higher level (Foynes *et al.* 2003). Other work has shown that the copy number of the plasmid carried by an *S. typhimurium aro* mutant is inversely proportional to the ability of the bacteria to colonise mice, presumably due to the metabolic burden of maintaining a high copy number plasmid (Bell *et al.* 1990).

1.5.4 DNA Vaccines

1.5.4.1 Background and Development

A relatively novel approach to inducing immune responses involves the administration of naked DNA that encodes the vaccine antigens of interest, rather than of the antigens themselves. This system relies on the use of a eukaryotic promoter to drive expression of the DNA encoded antigen within the transfected host cell. First described in 1990 (Wolff *et al.* 1990), its application to vaccine design resulted from the observation that specific antibodies could be generated to the antigen encoded by the DNA following intramuscular administration (Tang *et al.* 1992). These findings were mirrored by later work (Williams *et al.* 1992), which used an alternative method of vaccination; ballistic delivery of DNA into the dermis

using a gene gun (see below). These initial studies have led to a massive expansion of research in this area. Work to date has investigated the possibilities of using DNA to act as a vaccine in humans and animals against a variety of diseases. Many approaches have been considered and a number of human clinical trials have been completed. However, these have demonstrated that DNA vaccines need to be used in combination with other vaccine strategies to generate immunity. Examples include vaccines for malaria (Epstein *et al.* 2004; Moorthy *et al.* 2004), HIV-1 (Mwau *et al.* 2004), and various cancers (Tagawa *et al.* 2003).

DNA vaccines require a similar investment, in terms of development of the technology, to other forms of vaccines. However, production of the vaccine itself is much simpler and therefore potentially cheaper; there is no requirement for a complex purification of recombinant protein from bacteria (thus ensuring there is no potential for residual toxicity due to co-purified contaminants). DNA is easily isolated in a highly pure form, and can be dried out for storage. Transport of lyophilised DNA is simple; it requires no refrigeration and is stable for long periods of time, unlike protein vaccines, which commonly require refrigeration.

1.5.4.2 Generation of Immune Responses to DNA Vaccines

A summary of the generation of immune responses following DNA vaccination is shown in Figure 1-7. DNA vaccination has been shown to elicit $CD4^+$ T cell, $CD8^+$ T cell and B cell responses. However, the precise mechanisms by which these responses are generated are not clear, probably depending on which transfected cells are involved in the expression of antigen and acting as professional APCs to $CD4^+$ T helper cells.

Initially, DNA is taken up into the cells of the vaccine recipient and translocated into the nucleus by as yet undefined mechanisms. From there, the

DNA is transcribed into mRNA and translated into protein by exactly the same process that cellular proteins are manufactured. Translation is followed by protein modification. Such proteins can then be processed and presented on the surface of the cell in the context of MHC class I molecules. This can lead to the activation of $CD8^+$ T cells. In addition, specialist APCs can present vaccine antigen (either following direct transfection of the APC, or internalisation of antigen expressed and released following death of by other transfected cells). Antigen presentation by APCs can be in the context of MHC class I as above, or class II, potentiating the activation of $CD4^+$ T cells. Finally, B cells can also be activated following an encounter with vaccine antigen, although given the typically low humoral responses to DNA vaccines administered alone, this is thought to occur at a low level.

Currently the precise mechanism by which professional APCs could acquire DNA-expressed antigens is unknown. It is possible that transfected muscle cells or keratinocytes could produce antigen that is picked-up by APCs. Alternatively, cells at the site of vaccination (e.g., Langerhans cells – LCs – in the dermis) could undergo direct transfection, or be transfected by DNA that moved from the target site through the blood vessels or lymphatics. Since neither muscle cells nor keratinocytes constitutively express MHC class II or the co-stimulatory molecules required to activate T-helper (Th) cells, their role in antigen processing and presentation may not be as APCs, but they may still have a significant role in the expression of the encoded antigen.

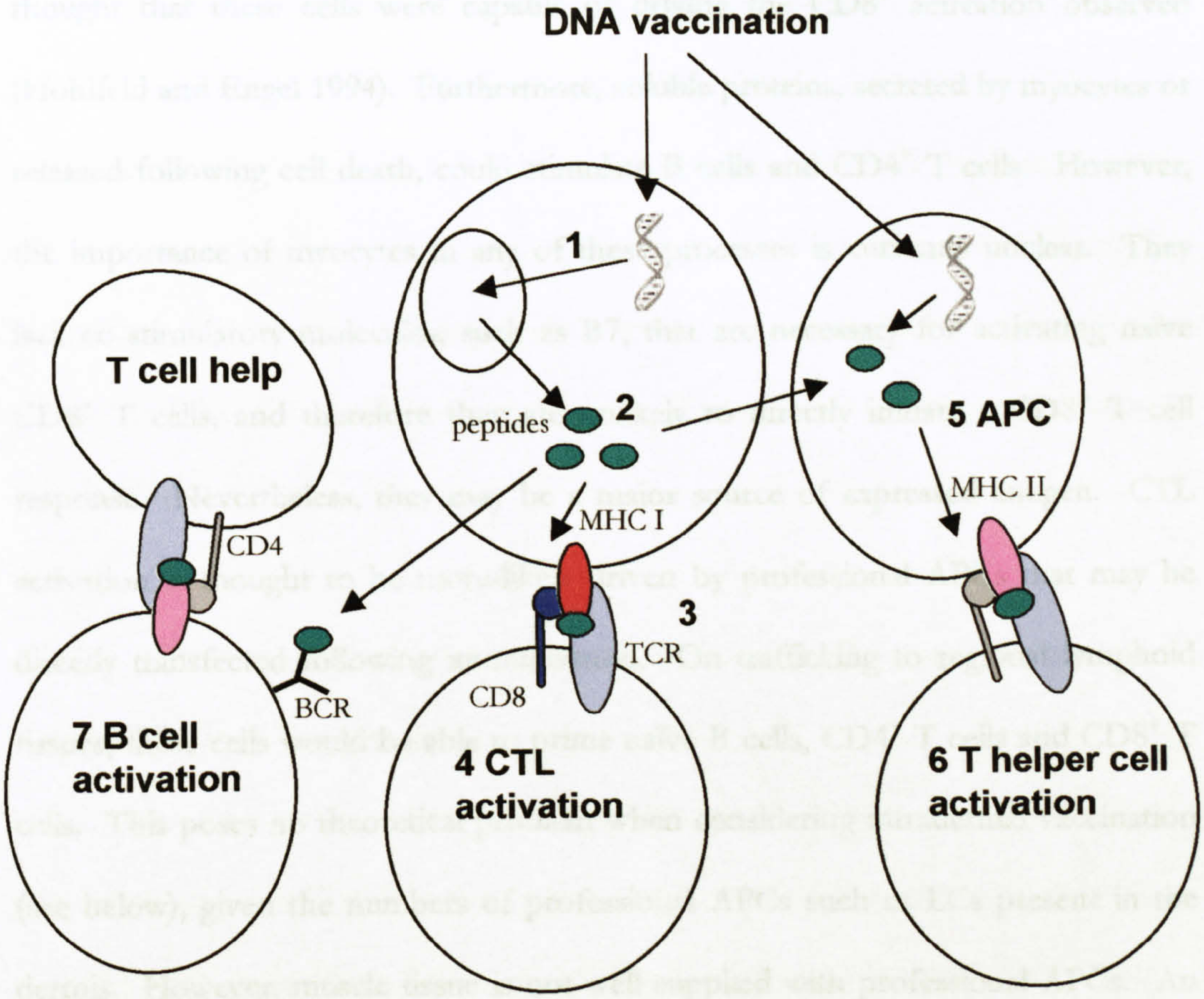


Figure 1-7: The development of immune responses following DNA vaccination

Following vaccination, DNA is taken up into the nucleus (1). DNA is translated into mRNA and the encoded antigens are then expressed in the cytoplasm by the normal cellular mechanism (2). Following expression, the antigens will be processed and presented on the cell surface in the context of MHC class I molecules (endogenous pathway). The MHC-antigen complex may then be recognised by a specific $CD8^+$ T cell (3), which is then activated and matures, resulting in proliferation and cytokine secretion (4). Activated CTLs will initiate apoptosis in any cells subsequently encountered presenting MHC-I-antigen complexes, which bind to the TCR in conjunction with a secondary signal. Antigens from a DNA vaccine may be encountered by professional APCs (5). These can be taken up into the cell by endocytosis and processed through the exogenous pathway, whereby epitopes are presented on the cell surface bound to MHC class II molecules. Subsequent interactions with naïve $CD4^+$ Th cells will then result in their activation, characterised by proliferation and secretion of cytokines in order to co-ordinate the cellular and humoral immune responses (6). If a B cell encounters an antigen to which its receptors can bind, it will begin to secrete antibody. A T helper cell with the same specificity can induce a B cell to mature into a plasma cell, whilst proliferating and undergoing class switch from IgM production to other classes including IgG (7).

It is known that myocytes can express vaccine antigen following transfection *in vivo*, and that they constitutively express MHC class I. Therefore it was initially thought that these cells were capable of driving the $CD8^+$ activation observed (Hohlfeld and Engel 1994). Furthermore, soluble proteins, secreted by myocytes or released following cell death, could stimulate B cells and $CD4^+$ T cells. However, the importance of myocytes in any of these processes is currently unclear. They lack co-stimulatory molecules, such as B7, that are necessary for activating naïve $CD8^+$ T cells, and therefore they are unlikely to directly initiate a $CD8^+$ T cell response. Nevertheless, they may be a major source of expressed antigen. CTL activation is thought to be more-likely driven by professional APCs that may be directly transfected following immunisation. On trafficking to regional lymphoid tissues, these cells would be able to prime naïve B cells, $CD4^+$ T cells and $CD8^+$ T cells. This poses no theoretical problem when considering intradermal vaccination (see below), given the numbers of professional APCs such as LCs present in the dermis. However, muscle tissue is not well supplied with professional APCs. An alternative possibility is that the tissue damage caused by i.m. vaccination results in the recruitment of APCs to the muscle. These cells could then internalise DNA and express and present antigen themselves, or provide secondary signals for antigen presenting muscle cells; it is thought that antigen presentation and co-stimulation do not have to be provided by the same cell (Kundig *et al.* 1995).

Some of the mechanisms that may be responsible for this process are outlined above in section 1.4.1.3, Cross Presentation. It is still uncertain precisely which cell types are involved in the generation of the activated T cells, but there is some evidence to suggest that the immune response may be modulated by altering the cellular localisation of expressed protein and the immunisation route (Morel *et al.* 2004).

Low antibody responses seen in response to some DNA vaccines suggest that DNA vaccines drive limited B cell activation. This is probably due to the low levels of antigen expressed by transfected cells in comparison to the quantity delivered by e.g. recombinant protein vaccination (Davis and McCluskie 1999).

After administration, it has been demonstrated that antigen expression can continue for a significant time period (Wolff *et al.* 1990). This low but ongoing expression of antigen continuously augments the immune response and it has been conjectured that this similarity to natural infection enhances the response (Donnelly *et al.* 1997).

1.5.4.3 Factors Affecting the Nature of the Immune Response Generated

Level of Antigen Expression

There is a direct correlation between the expression level of secreted proteins encoded by DNA vaccines and humoral responses, although there is no such correlation for intracellular proteins (Barry and Johnston 1997). The level of expression is also thought to have an effect on the induction of CTLs. However whilst increases up to a certain level gave an increased response, high doses actually resulted in a decreased CTL response (Barry and Johnston 1997). It maybe that higher levels of expression resulted in more rapid killing of transfected cells, curtailing the activation of CTLs.

Nature of the Antigen

The post-transcriptional localisation of antigen expressed following DNA vaccination has a major impact on the subsequent immune response. The effect of localisation was dissected by immunisation using DNA expressing cytoplasmic, secreted or transmembrane ovalbumin. Gene gun immunisation with cytoplasmic

or transmembrane protein led to strong ovalbumin-specific CTL responses, but not following immunisation with secretory protein. In contrast, i.m. immunisation with DNA expressing the secretory or transmembrane protein led to potent CTL whilst vaccination with the cytoplasmic form was ineffective. OVA-specific antibodies were detected following gene gun immunisation with all three constructs, whereas only the secretory construct induced antibody production following i.m. immunisation (Morel *et al.* 2004). These results demonstrate that the localisation of the protein is central to the nature of the response generated and indeed whether any response is elicited and that CTL and antibody responses are induced by different mechanisms.

Vector and Promoter Selection

The plasmid vector is responsible for directing transcription of the encoded antigen within cells transfected during vaccination. The backbone of the vector can be important due to the presence of immunostimulatory CpG motifs (see below). The other main significant feature is the promoter. The human cytomegalovirus (CMV) immediate early promoter is the most commonly used as it has been shown to drive high levels of antigen expression in murine skeletal muscle *in vivo*, following either gene gun or i.m. vaccination (Cheng *et al.* 1993; Manthorpe *et al.* 1993).

Immunostimulatory CpG Motifs

Bacterial plasmids used in DNA vaccination differ from eukaryotic DNA in several subtle but important characteristics. Firstly, bacterial CpG motifs (cytidine – phosphate – guanidine dinucleotides) are unmethylated, unlike those found in eukaryotic DNA. Secondly, these motifs are present at the frequency predicted by chance (1 in 16 dinucleotides) in bacterial DNA, whereas they are far less common

in eukaryotic DNA. These differences have no biological effect on the function of the DNA to store, replicate and transcribe genetic information, but can be exploited by the immune system to differentiate between mammalian DNA and bacterial DNA (Krieg 2002). In particular these differences are recognised by TLR-9 found on the surface of immune cells (Brown and Corral 2002). The stimulatory activity of the DNA motifs depends on the precise nucleotide sequence surrounding each motif (Krieg *et al.* 1999).

Recognition of unmethylated CpGs is mainly mediated by DCs, since they express high levels of TLR-9. However, the activity of B cells, macrophages and NK cells are all directly affected to some degree. In B cells, recognition strongly enhances antigen-specific activation by upregulating co-stimulatory molecule expression, and can drive T cell-independent class switching. Secretion of IL-6, IL-10 and immunoglobulin are all upregulated. Innate effector mechanisms are enhanced, such as phagocytosis and the release of antimicrobial reactive oxygen species in macrophages, and NK cytotoxicity and IFN-γ secretion are increased. T cells are not-directly affected, but can be more readily activated due to the changes in APCs (Cowdery *et al.* 1996; Krieg *et al.* 1999; Brown and Corral 2002).

Recently, synthetic oligodeoxynuleotides, containing immunostimulatory CpG motifs, have been used as adjuvants in various vaccine strategies. They drive Th1 responses in mice and primates, probably due to the induction of IL-12 and IFN-γ (Krieg *et al.* 1999). In DNA and protein vaccines, CpGs have been shown to be effective at increasing antibody production, both systemically and at mucosal surfaces (McCluskie *et al.* 2001; McCluskie *et al.* 2002; Klinman 2003).

Delivery Route – Ballistic versus Intramuscular Vaccination

DNA vaccination offers a degree of flexibility in terms of the quality of immune response generated, depending on the means of administration of the vaccine. It has been shown in many instances that immunisation by i.m. vaccination can result in an immune response dominated by $CD4^+$ cells of the Th1 phenotype. However, ballistic delivery of DNA into the dermis can elicit a Th2 dominated response. Ballistic delivery involves attaching plasmid DNA to microscopic gold particles, which are fired into the skin under pneumatic pressure, using a device known as a 'gene gun'. The different immune responses generated to i.m. and gene gun DNA vaccination have been noted in several studies (Zlei 2002; Zhu *et al.* 2004).

Induction of Th1 responses following i.m. vaccination has been partly explained by the presence of immunostimulatory CpG motifs on plasmid DNA. In fact, although gene gun vaccination has been shown to preferentially induce Th2 immunity this bias can be overcome by co-administering the vaccine DNA with large quantities of CpG motifs (Schirmbeck and Reimann 2001; Zhou *et al.* 2003). This potential to overcome Th2 bias could be exploited in the therapeutic vaccination of chronic diseases where an ineffective Th2 immune response dominates.

The inhibitory feedback between Th1 and Th2 cytokines can make immunomodulation of existing responses difficult, since an existing Th2 cytokine milieu around naïve $CD4^+$ T cells will hinder their development into Th1 cells. Thus vaccination strategies that induce strong Th1 responses and specifically prime $CD8^+$ T cell responses, such as i.m. DNA vaccines, may be necessary for effective therapeutic vaccination (Zajac *et al.* 1998). Immunomodulation is not only important in infectious disease, it can also be used to treat cancers and

autoimmunity (Prud'homme *et al.* 2001). However, this potential may not be as apparent as in mouse models. Although human memory T cells restimulated in the appropriate cytokine milieu may be able to switch Th phenotype, mouse memory cells do not share this ability (Sallusto *et al.* 2004).

1.5.5 Prime-Boost Strategies

Conventional vaccines are often successful at generating effective antibody responses that can control or prevent a wide range of infections. However, as discussed above (see section 1.2.2, The Requirement for New Vaccines), there remain many diseases for which such a response is not sufficiently protective. These include tuberculosis, HIV and malaria, currently the three infectious diseases responsible for the greatest numbers of deaths world-wide. All three are intracellular pathogens, and cannot be cleared by an immune response lacking a potent cell mediated component. Homologous boosting (delivering multiple doses of the same vaccine formulation) can often increase humoral responses to the level where they are protective, such as with the recombinant HBsAg of the HBV vaccine. However, the strategy of repeatedly delivering the same vaccine is not effective at increasing the magnitude of $CD8^+$ T cell responses.

The advent of DNA vaccination was treated with enthusiasm as the ability to induce strong cell-mediated immune responses offered the tantalising prospect of effective vaccination against such intransigent diseases. In reality, DNA vaccination has not yet lived up to initial expectations. A logical progression was therefore to combine different vaccination strategies in heterologous prime-boost regimes. Sequential vaccination with the same antigen employing different strategies of

delivery has been shown to improve immunity by the two vaccines working synergistically together (McShane 2002).

One of the aims of this project is to examine the effects of boosting a DNA prime vaccination with different heterologous boosts and determine the extent to which responses are enhanced. Whilst many strategies are able to prime cell mediated immunity, few have been shown to boost cellular responses effectively. The response after a primary vaccination generally focuses on a few immunodominant epitopes of an antigen. It is thought that boosting the response with the same antigen allows a preferential expansion of pre-existing memory T cells to these epitopes. When a different vector is employed, the further development of responses against the first vector is avoided. This may prevent a 'dilution' of the response, instead allowing the immune system to focus on the vaccine antigen itself (Woodland 2004).

The successful use of prime-boost vaccination strategies is dependent on the ability of the boost vaccine to enhance the primed immune response. For this to be achieved the boost needs to be given proximally to that of the prime to ensure that memory T and B cells residing in local draining lymph nodes are stimulated. In the case of attenuated vectors, an association with immune cells (such as the uptake by macrophages of *Salmonella*) may be an advantage since this could direct antigen more efficiently to lymphoid tissues and memory lymphocytes (Ramshaw and Ramsay 2000).

There are various examples of prime-boost vaccination displaying synergistically improved responses (Kanellos *et al.* 2000; Estcourt *et al.* 2002; McCluskie *et al.* 2002). One in particular is the increased efficacy of BCG vaccination against tuberculosis when vaccination with the bacteria was enhanced by prior administration of a DNA vaccine. The DNA vaccine alone had little effect.

However, DNA prime followed by a BCG boost offered improved efficacy over BCG alone in an aerosol challenge model. Protection was mediated in part by CTL activity (McShane 2002).

Application of effective prime boost strategies has not only been suggested for the development of new prophylactic vaccines but also in the development of therapeutic vaccines. This type of vaccine would be used in the treatment of a chronic infection such as Hepatitis B in which activation of a specific arm of the immune system may serve to clear the disease.

1.6 Hepatitis B Virus

HBV is an enveloped virus with an icosahedral core and double-stranded circular DNA genome. The virus invades and replicates within the liver cells of the human host. Following replication, assembled virus particles bud from the cell surface (Ganem and Schneider 2001).

The virus can be passed on through sexual contact and body fluids such as blood, including untreated blood products. The high viral load in the blood of those infected renders it highly infectious through contaminated needles. Acutely infected adults can normally clear the virus. However, chronic infection is a problem with recent estimates of 350 million people infected world-wide (WHO 1996). As about 25% of chronic carriers (1 million people each year) will die of chronic active hepatitis, cirrhosis or primary liver cancer there is an obvious need for new treatments to be developed.

Although, a useful prophylactic vaccine exists, based on parenteral delivery of recombinant surface antigen expressed in yeast, it is not effective as a therapeutic vaccine in chronically infected patients (WHO 1996).

1.6.1 Host-Pathogen Interactions

One area of much interest over the last 5 years is the potential to modulate the immune response to HBV to encourage clearance of the virus as persistence is believed to be due, at least in part, to modulation of the immune response by viral antigens. Patients who suffer from a self limiting disease usually display evidence of a strong, broad Th1 response (Chisari and Ferrari 1995; Guidotti *et al.* 1996). In contrast, chronically infected patients display a strong humoral response to the virus but usually have weak CTL activity against only a few viral epitopes. This does not imply that the Th2 response is ineffectual as there is clinical evidence that significant levels of circulating antibody are also necessary for clearance and to protect against re-infection (Milich and McLachlan 1986; Milich *et al.* 1995). However, these humoral responses alone are not sufficient for clearance of an established infection.

Interestingly, there is evidence that the virus itself plays a role in modulating the immune response to prevent effective clearance through the core antigen. The gene for this protein contains two in-phase start codons, one of which leads to the transcription of HBcAg, the other a protein that is processed in the ER and secreted from the cell as the HBeAg. This antigen, although largely identical to core in sequence, is a soluble protein, which unlike the HBcAg does not self assemble to form particles. It has been demonstrated that this non-particulate form of the antigen, is effective at stimulating a Th2 response which is less effective at clearing the virus than the Th1 response induced by the particulate antigen (Milich *et al.* 1997). The high level of the HBeAg observed in the blood stream of chronic patients effectively swamps the immune system with antigen (Milich *et al.* 1998). This may result in the lack of a broad CTL response, which has been proposed as

the reason why the virus is ineffectively cleared, and thus represents a sophisticated evolutionary strategy, on the part of the virus, to enable chronic carriage and therefore increase the likelihood of dissemination to other hosts.

1.6.2 Therapeutic Vaccination and HBV Infection

A possible hypothesis for the successful clearance of HBV infection is that hepatocytes infected with the virus present viral antigens to CTL which then trigger apoptosis in these cells. On activation, however, the CTL begin to secrete Th1 cytokines such as IL-12 and IFN-γ (Rossol *et al.* 1997). The latter cytokine induces an antiviral state in hepatocytes, resulting in degradation of viral RNA, abrogation of virus assembly and preventing future infection of cells (Guidotti *et al.* 1999). As the Th1 response brings the population of infected hepatocytes under control, B cells produce neutralising antibody that mops up circulating virus, preventing re-infection. Conversely, it may be that the poor activation of CTLs and Th2-like immune response in chronic carriers prevents them from clearing the virus (Chisari and Ferrari 1995; Guidotti *et al.* 1996).

If this hypothesis is correct, it seems plausible that efforts aimed at curing chronically infected patients should induce a Th1 response. Indeed, current moderately effective treatments, such as interferon (IFN) and ribavirin therapy, are effective at raising Th1 responses (Hultgren *et al.* 1998; Michel *et al.* 2001).

Attempts have been made to use DNA vaccination in human patients to modulate immune responses to chronic HBV infection. They have been partially effective; inducing CTL responses and reducing viral replication (Michel *et al.* 2001), but not leading to full clearance. It is possible that these results could be improved using combined vaccination/antiviral therapy or by employing a heterologous

prime-boost vaccination strategy. One difficulty with attempting to induce Th1 immunity through vaccination has been the historical lack of safe, effective Th1 adjuvants (Moingeon *et al.* 2001). However, DNA vaccines contain immunostimulatory CpG motifs (described above) that strongly drive Th1 responses. It is therefore possible that DNA vaccination will become central to therapeutic vaccination strategies.

1.6.3 Structure and Immunogenicity of the Core Antigen

The model antigen used for the purposes of this project was the hepatitis B core antigen (HBcAg), the capsid protein of the hepatitis B virus. This monomeric protein of approximately 21kDa in size (as shown in Figure 1-8) is capable of self-assembly into large particles when present in sufficient concentrations. The assembled particle is shown in Figure 1-9. The antigen was selected as a model because it is highly immunogenic and it provides a unique opportunity to consider the impact of prime boost strategies on particulate rather than soluble antigens, as described previously in this laboratory (Zlei 2002). The reason for this comparison is the different nature of particulate and soluble antigen processing (see section 1.4.1). Particulate antigens are presented with an efficiency three to four orders magnitude greater than that of soluble antigen (Kovacsovics-Bankowski *et al.* 1993; Harding and Song 1994).

The particle itself is made up of many dimers of the core protein. During natural infection, it forms the capsid in which viral DNA and a DNA polymerase enzyme are packaged. Although the protein consists of 183 amino acids, only the N-terminal 140 amino acids are required for generation of the particulate structure, although particle stability is increased by inclusion of a cysteine at position 183

which forms an internal disulphide bridge. The remaining 43 amino acids provide the nucleic acid binding region required for encapsulation of the viral genome. The capsid particles can consist of either 180 or 240 subunits, forming particles of 320 and 360 Angstrom in width, respectively (Wynne *et al.* 1999).

The polymers are made up of many pairs of individual polypeptides. These dimers are highly stable and resistant to full denaturation. This is believed to be due to interactions between the α-helices of the c1 loop (see below) (Bottcher *et al.* 1997), and the extensive hydrophobic core of the protein. A cysteine-cysteine disulphide bridge is formed between the α-helices in dimers, but is not necessary for dimer or capsid formation (Wynne *et al.* 1999).

The major structural and immunological feature of the capsid is a surface spike. In the monomer, two consecutive α-helices, from amino acid 50-73 and 79-110, form a hairpin structure known as the c1 loop (Wynne *et al.* 1999). These associate in the dimer to form a 4-helix bundle, which protrudes from the surface of the capsid by about 25 Angstrom (Conway *et al.* 1997).

HBcAg contains several highly immunogenic T and B cell epitopes against which strong immune responses can be raised (Milich *et al.* 1995). It can function as a T cell-independent antigen, because of its ability to activate naïve B cells directly by the cross linking of multiple BCRs, resulting in the induction of B7 co-stimulatory molecules (Milich *et al.* 1997). This does not, however, prevent the HBcAg from promoting T helper responses, therefore it is both a T cell-dependent and T cell-independent antigen (Milich and McLachlan 1986). The principle antigenic site of the protein is found at amino acids 78 to 82 and coincides with the tip of the surface spike (Bottcher *et al.* 1997) (see Figure 1-8).

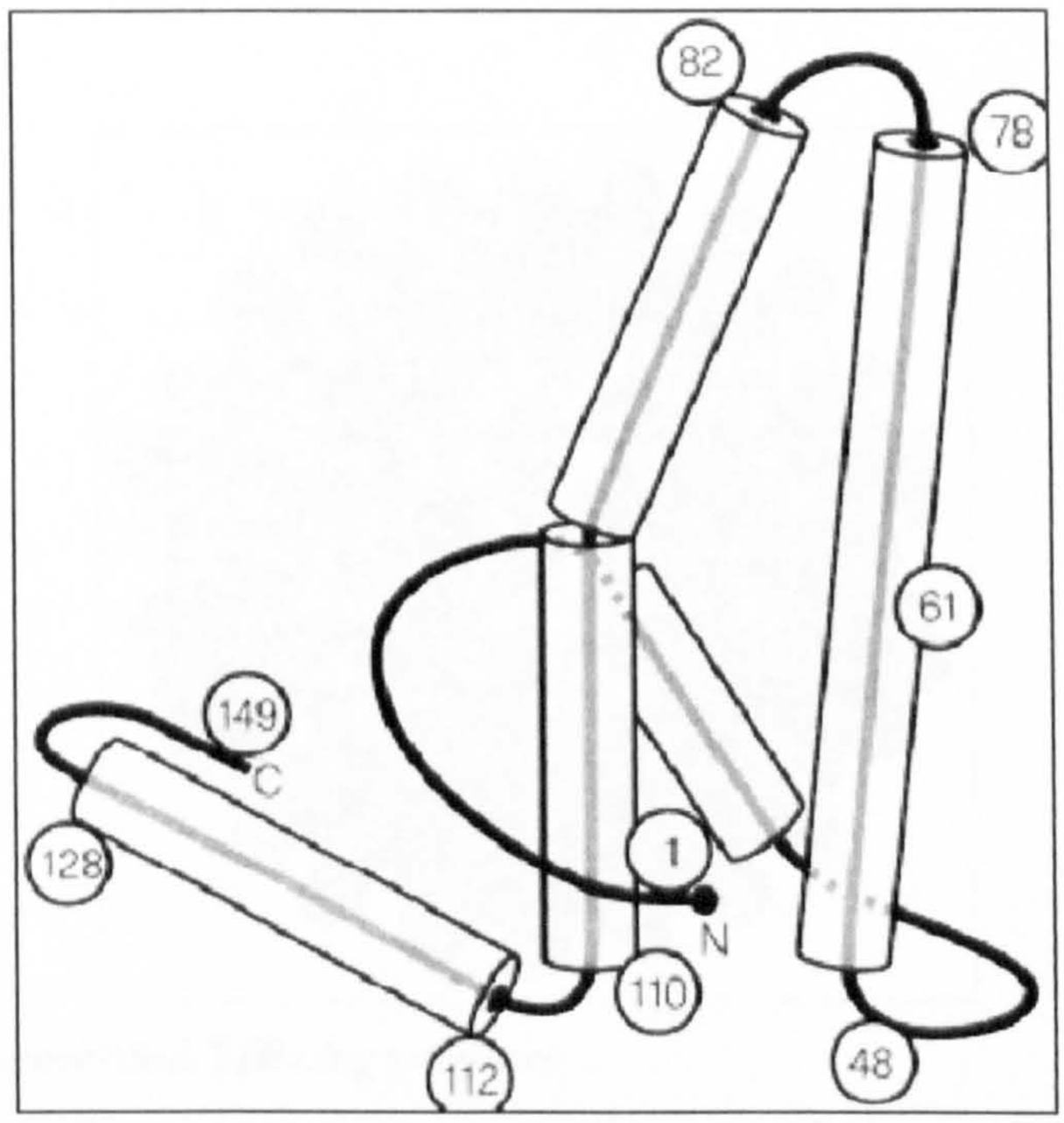


Figure 1-8: Three-dimensional layout of the truncated core protein monomer

The numbers represent the amino acid positions of principles features, i.e. C and N terminal residues, start and end of α helix regions and cysteine residues (Bottcher *et al.* 1997).

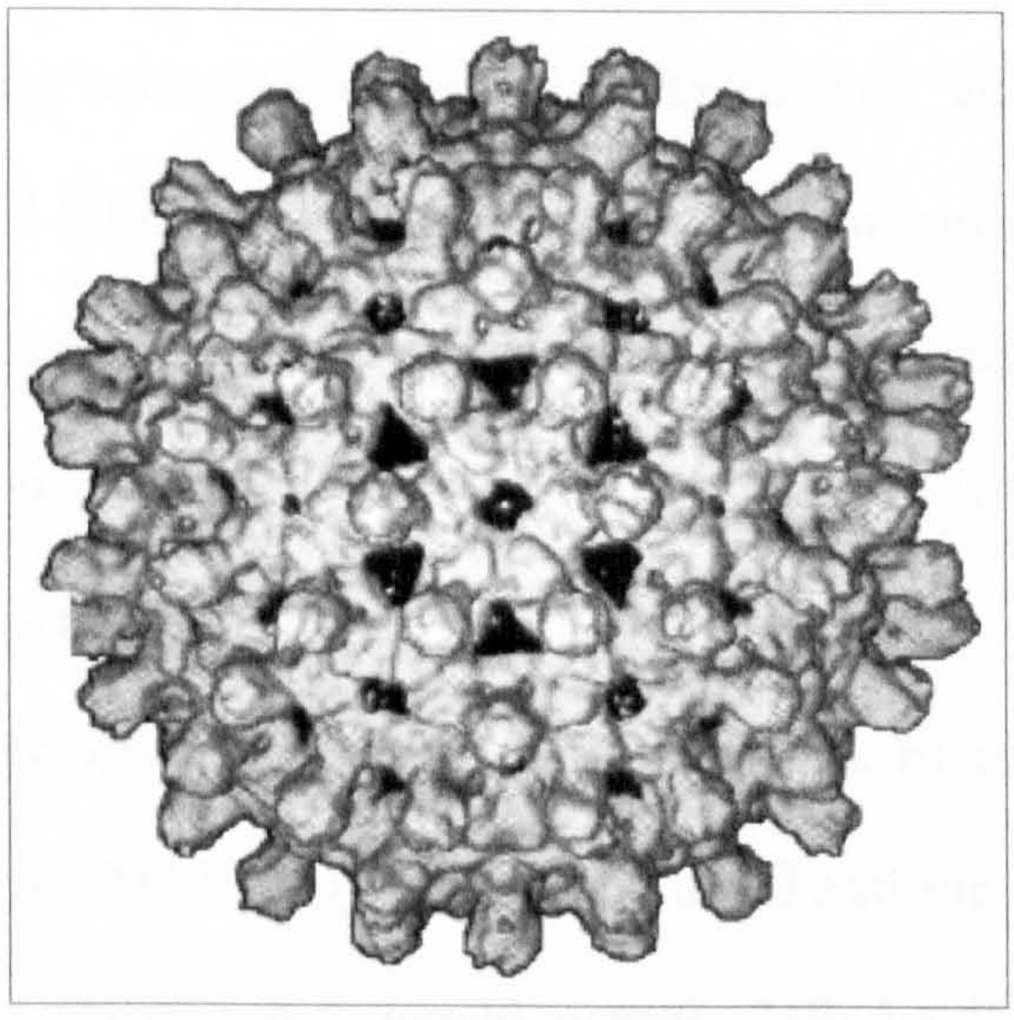


Figure 1-9: Assembled HBcAg polymer

The assembled HBcAg particle, determined at a resolution of 9 Angstroms from cryo-electron micrographs (Conway *et al.* 1997).

PROJECT AIMS

This project was designed to fulfil two main objectives.

- The first was to characterise and compare the immune responses developed following intra-muscular (i.m.) and gene gun delivery of a DNA vaccine consisting of a plasmid expressing HBcAg.
- The second was to determine the effect of boosting the primary response to DNA vaccination through the use of heterologous boost strategies.

The boosting strategies examined included i.m. administration of the recombinant purified HBcAg alone , intra-nasal (i.n.) administration of the purified antigen in combination with mucosal adjuvant LT and intra-gastric (i.g.) administration of an attenuated bacterial vector (*S. typhimurium* BRD 509) that expresses the HBcAg *in vivo.*

In order to complete this work, a plasmid capable of expressing the HBcAg in eukaryotic cells, pcDNA3.1/core, was constructed and the *Salmonella* strain was transformed with a plasmid that would express HBcAg following invasion of mammalian cells.

To characterise the humoral and cell-mediated immune responses induced by the different vaccination strategies, assays were developed which allowed measurement of HBcAg specific serum and mucosal antibody production, $CD4^+$ T cell proliferation and $CD8^+$ T cell activation and killing capacity.

2 MATERIALS AND METHODS

2.1 Microbiology

2.1.1 Bacterial Stocks

Master stocks of bacteria were maintained on microbank beads at -80°C. Working stocks were kept in 50% glycerol/Luria Bertani (LB) broth at -80°C. Bacteria were streaked out onto LB agar plates containing the appropriate antibiotics and grown at 37°C to confirm resistance and colony morphology before use.

2.1.2 Transformation of Competent *Escherichia coli* by Heat Shock

E. coli XL10 Gold® competent bacteria (Stratagene) were taken from storage at -80°C and thawed on ice. 4µl of 1.42M β-mercapto-ethanol was added to bacteria, which were incubated on ice with occasional gentle mixing for 10 minutes. To transform the bacteria, between 0.1 and 50ng of experimental DNA was added, mixed gently and incubated on ice for 30 minutes. The bacteria were heat pulsed for thirty seconds in a water bath at 42°C, then incubated on ice for two minutes. 0.9ml of preheated (37°C) LB broth was added to each tube and the tubes incubated for 1 hour at 37°C in a rotary shaker (~200rpm). The bacteria were then plated onto agar plates in the presence or absence of the appropriate antibiotics and incubated overnight at 37°C.

The ratio of colonies on antibiotic and antibiotic free plates gave an estimate of transformation efficiency. Colonies were screened by PCR or by restriction analysis (see below) to determine if the transformation was successful.

2.1.3 Making Competent *Salmonella typhimurium*

A 5ml overnight starter culture of *S. typhimurium* LB5010 (*gal*E) was used to inoculate a 500ml flask of media. The culture was grown to mid logarithmic phase (OD_{650} of ~0.5) and then the bacteria concentrated by centrifugation at 4°C, at 10,000g. The resultant pellet was resuspended in 200ml TFB1 (30mM sodium acetate, 100mM RbCl, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% glycerol in distilled water) and incubated on ice for 5 minutes. The bacteria were then pelleted as described previously and finally resuspended in 20ml of TFB2 (10mM RbCl, 75mM CaCl2, 10mM MES, 15% glycerol in distilled water). The competent cells were then aliquoted into microfuge tubes and stored at -80°C. When required, these bacteria were transformed with plasmid as described above.

2.1.4 Creation of Recombinant Bacteriophage P22/pGA-1

D-galactose was added, at a final concentration of 0.04%, to an overnight culture of *S. typhimurium* LB5010, which had been previously transformed with the plasmid pGA-1 using the method described above. The culture was then incubated for a further 45 minutes to allow uptake of D-galactose and synthesis of smooth LPS, making the bacteria susceptible to bacteriophage P22 infection.

During this time, buffer T2 (66mM KH_2PH_4, 69mM NaCl, 29mM K_2SO_4, 17mM Na_2HPO_4, 1mM $MgSO_4$, 55µM CaCl.$6H_20$, 0.01% gelatin in 1L distilled H_20)

was used to dilute the P22 stock into 10-fold serial dilutions. 100µl of bacterial culture was added to tubes containing 10µl of virus for each dilution. The tubes were incubated for 1 hour statically at 37°C to allow the phage to infect the bacteria.

Following infection, previously melted top agar (1g tryptone, 0.8g agar, 140mM NaCl in 100ml distilled H_20) was cooled to 48°C and 3.5ml was added to each bijou. The contents were poured evenly over an agar plate. Plates were incubated for 5-6 hours at 37°C until plaques were visible. A sterile microscope slide was used to scrape off the top agar layer. The virus was then recovered from this top agar by addition of 3ml T2 buffer and 200µl chloroform to the agar (to kill any remaining live bacteria) to the agar. The tubes were incubated for 30 minutes at 37°C to release the bacteriophage and then centrifuged for 5 minutes at 1000g. The supernatant, containing the recombinant phages, was recovered and stored in a glass bijou, with 50µl fresh chloroform, at 4°C.

2.1.5 Transduction of *S. typhimurium* BRD509 with Bacteriophage P22

The recombinant virus was then used to transform the vaccine strain (*S. typhimurium* BRD509, attenuated by deletion of two *Aro* genes (Strugnell *et al.* 1992), see Introduction section 1.5.3.4. Overnight cultures of this strain were aliquoted into microfuge tubes to which different volumes of the supernatant containing the phage were added. These were incubated at 37°C for 30 minutes to allow infection and then spread onto LB plates containing ampicillin/5mM EGTA. The presence of EGTA in the media prevents the virus completing its life cycle, so the infected bacteria are not lysed by replicating phage.

The bacteria were re-streaked onto LB plates containing ampicillin and EGTA after overnight growth, to ensure only those bacteria into which the bacteriophage had successfully passed the plasmid of interest would survive.

2.1.6 *Salmonella* Viable Counts

Viable counts of *Salmonella* were made to accurately determine the relationship between OD and concentration of bacteria for the purposes of vaccination and also to determine the numbers of bacteria recovered from tissue of previously inoculated mice. These were performed by making 10-fold serial dilutions of the bacterial suspension and plating 10ul volumes, in triplicate, onto quadrants of agar plates. The plates were incubated overnight at 37°C. The plates containing the highest concentration of colonies that could be distinguished separately were counted and the colony numbers recorded.

2.2 DNA Manipulation and Preparation

2.2.1 Plasmids

2.2.1.1 pGA-1

This plasmid, kindly provided by Medevapharma PLC, was created from pPV404. The plasmid encodes the HBcAg, under the control of the *tac* promoter. In pGA-1 the *tac* promoter is replaced by the anaerobically activated *nirB* promoter, which induces translation under low oxygen concentration.

2.2.1.2 ptrc/core

The plasmid ptrc/core was also received from Medevapharma PLC. The core sequence was cloned from pGA-1 into a new plasmid, pKK233-2, in which expression is controlled by the *trc* promoter which is activated by addition of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the media.

2.2.1.3 pcDNA3.1+

This plasmid was obtained from Invitrogen. It has a eukaryotic (CMV) promoter and carries an ampicillin resistance gene. The plasmid is shown in Figure 2-1.

2.2.2 Small-Scale Preparation of Plasmid DNA

Small scale preparation of plasmid DNA from bacteria was carried out using the Qiagen miniprep kit. The protocol for this kit can be found at http://www1.qiagen.com/literature/protocols/QIAprepMiniprep.aspx.

The DNA was eluted in distilled H_2O (pre-heated to 60°C to increase elution efficiency). DNA was stored at -20°C for subsequent analysis and reactions.

2.2.3 Polymerase Chain Reaction

The reagents for use in the polymerase chain reaction (PCR) were combined as described in Table 2-1 - PCR master mix.

Template was acquired from plasmid DNA from a small-scale preparation of purified DNA (miniprep) or from bacterial lysates, prepared as follows. A freshly grown colony was picked from an agar plate using a sterile toothpick and the

bacteria added to 100µl of MB grade H_2O. The suspension was heated at 90°C for 10 minutes and then centrifuged for one minute to pellet the lysed bacteria. 10µl of the supernatant was added to the PCR mix for each reaction.

The PCR mix was aliquoted into individual sterile 200µl PCR tubes and the reaction was carried out using a Hybaid Genesprint PCR machine under the conditions described in Table 2-2 - PCR reaction parameters.

2.2.4 PCR Primers

The primers used in this project are outline in Table 2-3: Cloning and sequencing primers. All primers were synthesised by Invitrogen.

2.2.5 Nucleic Acid Endonuclease Restriction Digestion

The DNA to be digested was added to the reaction mix as described in Table 2-4. The total volume of the reaction mixture was then made to a final volume of 20µl with distilled H_2O. The reaction mixture was incubated for 1 hour at 37°C in a water bath and then the enzymes were inactivated by raising the temperature to 55°C for 5 minutes.

2.2.6 DNA Purification

Cleaning of DNA (isolating DNA from enzyme reaction mixtures and resuspending in H_2O) was carried out using the Qiagen PCR purification protocol. The method for this protocol can be found at: http://www1.qiagen.com/literature/protocols/QIAquickSpin.aspx

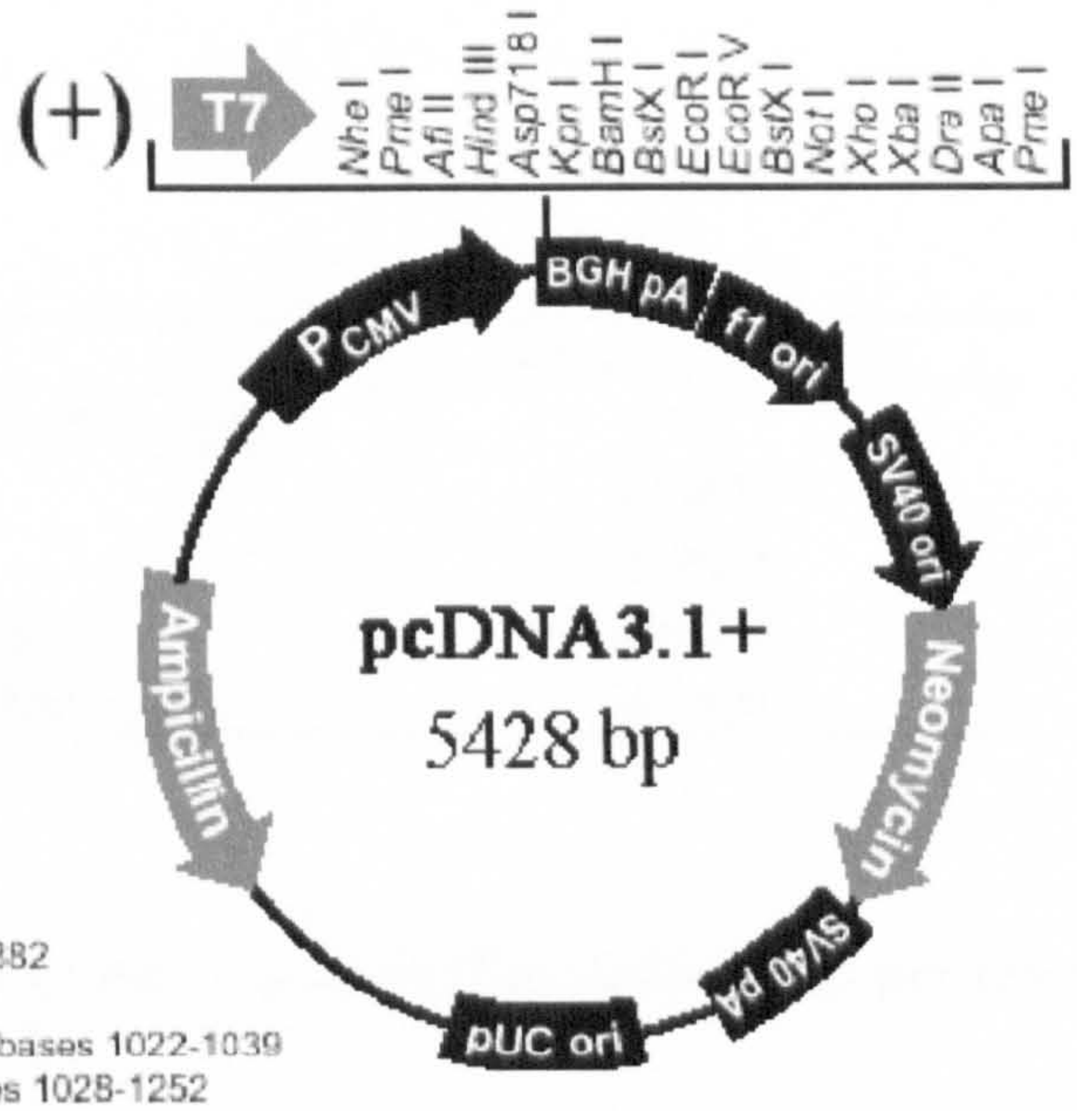


Figure 2-1: Schematic representation of the plasmid pcDNA3.1^{+}

This diagram, obtained from www.invitrogen.com, shows the main features of the plasmid including promoter (P_{CMV}), antibiotic resistance markers (Ampicillin and Neomycin) origin of replication (ori) and the restriction sites in the multiple cloning site.

Reagent	Final concentration
10x DNA polymerase buffer (Promega)	1x
$MgCl_2$	1.5mM
dNTPs	200µM
primers (Invitrogen)	400nM
Taq DNA polymerase (Promega)	2 units

Table 2-1 - PCR master mix

These reagents were made up to a total volume of 25µl distilled H_20 per reaction, including the template DNA.

Stage	Cycles	Temperature (°C)	Duration
1	1	94	60s
2	15-30	94	20s
		55 *	30s
		72	60s
3	1	72	5min
4	-	4	hold

Table 2-2 - PCR reaction parameters

* - the annealing step was modified depending on the predicted annealing temperatures of the primers.

Name	Function	Sense	Restriction sites	Sequence (start/stop codons underlined)
BJS 1	cloning	sense	*Hin*d III	TTG GGT GGA AGC TTA CCA TGG ACA TTG ACC C
BJS 2	cloning	anti sense	*Bam*H I	GCT TGG GGA TCC CTA ACA TTG AGA TTC CCT AG
BJS 4	sequencing (internal core)	sense	-	GCC TTC TGA CTT CTT TCC TTC C
BJS 5	sequencing (internal core)	anti sense	-	TCG GTC CCG TCG TCT AA
BJS 11	sequencing (CMV promoter)	sense	-	CGC AAA TGG GCG GTA GGC GTG
BJS 22	sequencing (pcDNA3.1 post MCS)	anti sense	-	TCT AGC AT TAG GTG ACA CTA TAG

Table 2-3: Cloning and sequencing primers

2.2.7 Agarose Gel Electrophoresis

Molecular biology-certified agarose (Bio-Rad) was mixed with TBE (90mM tris, 90mM borate, 2.5mM EDTA) at 2% (w/v) for separation of PCR products or digestion fragments <1000bp in size, or 1% for plasmids/fragments >1000bp. The agarose was melted, allowed to cool and then poured into a gel cast with 10µl of 1mg/ml ethidium bromide. Once the gel was set, DNA was mixed with DNA loading buffer (Sambrook *et al.* 1989) to give between 50 and 500ng DNA per well, in a volume of up to 25µl. 1Kb DNA ladder (Gibco) was run alongside the DNA samples at 1µg per lane, providing individual marker bands for accurate sizing of approximately 100ng. Gels were then run at 80V for at least 80 minutes (depending on the size of the fragments) and finally visualised under UV light.

2.2.8 Ethanol Precipitation of DNA

To precipitate DNA samples, two volumes of MB grade ethanol were added to an aqueous sample of DNA in a microfuge tube. 0.1 volumes of 3M pH5.2 sodium acetate was added. The mixture was frozen at -80°C for 30 minutes and then centrifuged at 10,000g for 30 minutes at 4°C. The tube was washed with 1ml 70% v/v ethanol, frozen and centrifuged again. The DNA could then be resuspended in the appropriate volume and medium.

Reaction component	Final concentration
DNA	200ng - 1μg per reaction
10x enzyme buffer	1x (composition varies with enzyme and manufacturer)
Acetylated BSA	2μg
Enzyme	1μl (10units)

Table 2-4: Restriction digest reaction mixture composition

2.2.9 Ligation of DNA Fragments

Pre-digested DNA fragments to be ligated together were added to the same microfuge tube, at the desired ratio (usually 3 : 1, insert : vector). The DNA was then ethanol precipitated and resuspended in the ligation mix (0.5µl T4 DNA ligase, 0.5µl 10x ligase buffer, 4µl distilled H_2O) and incubated overnight at 4°C. The ligated DNA was transformed into competent bacteria, which were screened for the recombined DNA by PCR.

2.2.10 DNA Quantification by UV Spectrophotometry

DNA was diluted in molecular biology grade water and measured in a spectrophotometer (Amersham Pharmacia biotech Genequant Pro RNA/DNA calculator) at wavelengths of 260 and 280nm. The concentration of double stranded DNA was calculated as fifty times the OD_{260}. OD_{280} gives a measure of the purity of the sample (Sambrook *et al.* 1989).

2.2.11 Large Scale Preparation of DNA for Vaccination

Large scale (~1mg) preparation of plasmid DNA from bacteria was carried out using the Qiagen megaprep kit. The protocol for use of this kit can be found at http://www1.qiagen.com/literature/protocols/QIAGENPlasmidPurification.aspx.

The DNA was resuspended in LPS-free Phosphate-buffered saline (PBS, 0.15M NaCl, 2.5mM KCl, 0.15M KH_2PO_4, 10mM Na_2HPO_4, pH7.3) for quantification and use in vaccinations.

2.2.12 Preparation of Gene Gun Cartridges for DNA Vaccination

2.2.12.1 Precipitation of DNA onto Gold Particles

Gene gun DNA cartridges were made with 2µg DNA and 500µg of 1µm diameter gold particles per cartridge. Due to losses during washing and binding the DNA, the final concentration is approximately 1µg DNA per cartridge. 100µg of DNA and 25mg of gold particles were added to 200µl 0.05M spermidine solution. 100µl of 1M calcium chloride was added slowly. The mixture was allowed to precipitate at room temperature for 10 minutes and then centrifuged briefly to pellet the gold. The supernatant was discarded and the pellet washed three times in 1ml MB grade 100% ethanol.

After the final wash, the pellet was resuspended in 3.3ml 0.05mg/ml poly vinyl pyrrolidone (PVP)/ethanol solution.

2.2.12.2 Loading DNA-Gold Suspension onto Gene Gun Cartridge Tubing

A 30 inch section of gene gun tubing was connected to the tubing prep station, and nitrogen allowed to flow through in order to clear the tubing. The DNA/gold suspension was vortexed thoroughly and aspirated into the tubing. The gold was allowed to settle out and the ethanol withdrawn slowly to give the tube an even coating of DNA/gold.

The tubing was then left to rotate whilst drying in nitrogen (0.4L per minute) for 30 minutes. The tube was then removed from the station and sectioned into cartridges of 0.5 inches.

2.2.12.3 Assessment of DNA Loaded onto Cartridges

To quantify the amount of DNA on each cartridge, every 10th cartridge down the tube length was removed and the following analysis completed.

Using a sharp, large bore hypodermic needle, holes were punched in the base of 0.5µl microfuge tubes. These were then placed inside 1.5 ml microfuge tubes. One cartridge was placed into each of the small tubes. 50µl of 10mM tris HCl pH8.5 was slowly pipetted into the cartridge. The cartridges were left at room temperature for ~3 hours and then vortexed, the liquid spun out and collected, and the procedure repeated overnight at 4°C.

The two samples for each cartridge were pooled and the DNA quantified by UV spectrophotometry (see above).

2.2.13 Quantification of Lipopolysaccharide in DNA

The limulus amaebocyte lysate (LAL) assay allows the quantification of LPS in a sample in a kinetic assay by comparison against a standard curve. Reagents are from the QCL endotoxin kit (BioWhittiker, Cambrex). LPS-free pipette tips and tubes were used throughout.

The LPS standard was reconstituted in LPS-free water and vortexed for 10 minutes. Four tenfold, 1ml serial dilutions were made from this stock, from 50IU/ml to 0.05IU/ml, vortexing each sample thoroughly between dilutions. Appropriate serial dilutions of samples were made in the same way. Standards were added in duplicate to a 96 well plate (Costar 3590). Samples were added to four wells for each dilution to be tested. Two wells of each sample were then inoculated with spikes of 5IU from the standard samples. This ensures that no contamination

in the sample could interfere with the assay. Blanks were added to two wells and the plate incubated at 37°C for ten minutes.

When the samples were up to temperature, 100µl of alkaline phosphatase conjugated limulus lysate was added to each well of the assay plate and the OD_{405} measured every 2.5 minutes over a period of 90 minutes. The results were analysed using the program Kineticalc 4 (Bio-tek instruments).

2.3 Protein Analysis

2.3.1 Antigen and Peptides

HBcAg expressed by *Pichia pastoris* was purchased from AMS biotec (Cat. No. R3B601).

All peptides were synthesised by Sigma Genosys, to a purity of >95%, based on epitopes identified by the references described in Table 2-5, and modified to reflect the sequence of the HBcAg used in this thesis.

2.3.2 Polyacrylamide Gel Electrophoresis (PAGE)

2.3.2.1 Preparation and Running of Polyacrylamide Gels

All gels were prepared using the Bio-Rad miniprotein II PAGE apparatus. The lower 12.5% resolving gel was prepared as described in Table 2-6.

Peptide	Epitope	Sequence	Reference
HBcAg p120-140	H-2^b/H-2^d $CD4^+$	VSFGVWIRTPPAYRPPNAP IL	(Milich *et al.* 1987)
HBcAg 93-100	H-2^b $CD8^+$	MGLKIRQL	(Kuhrober *et al.* 1997)
HBcAg 87-95	H-2^d $CD8^+$	SYVNTNMGL	(Kuhrober *et al.* 1997)

Table 2-5: HBcAg oligopeptide sequences

The sequences of oligopeptides synthesised by Sigma-Genosis and used in *ex vivo* restimulation assays.

Component	Composition	Volume added (sufficient for 10ml total or 2 gels)
Distilled water	-	3.35ml
Separating buffer	1.5M Tris, 0.014M SDS, pH 8.8 with HCl	2.4ml
30% Acrylamide	Acrylamide/bis acrylamide 29:1	4.15ml
10% ammonium persulphate (APS)	0.44M	60µl
Tetra methyl ethylene diamine (TEMED)	-	5µl

Table 2-6: Composition of separating gel for PAGE

After polymerisation, a 4.5% stacking gel was added and combs inserted prior to polymerisation to create appropriately sized wells (Table 2-7).

After polymerisation of this layer, the comb was removed and the gel, still between the glass plates, removed from the casting rig and placed in the PAGE gel running apparatus. The gel was submerged in PAGE running buffer (25μM tris, 192μM glycine, 3.5μM SDS) and the wells washed out by pipetting.

Samples were prepared by mixing with equal volume of 2x protein sample buffer (0.125M Tris, 10% β-mercaptoethanol, 20% glycerol, 4% SDS, 0.1% bromophenol blue) and boiled for 5-10 minutes. They were centrifuged for approximately 5 seconds to remove insoluble protein/particulate debris and then loaded into the wells. 10μl Seeblue ladder (Invitrogen) was added to one well to provide size markers. Electrophoresis of the samples was then carried out at 200V for 50 minutes.

2.3.2.2 Staining of Polyacrylamide Gels

Once full protein separation had been completed, gels were carefully removed from glass plates and immersed in ~50ml Coomassie blue stain (1% Coomassie brilliant blue, 45% methanol, 10% glacial acetic acid) and left shaking gently overnight at room temperature. To remove the stain, the gel was washed several times in destain solution (45% methanol, 10% glacial acetic acid) until the background was colourless. For long term storage, gels were dried between cellophane layers using Gel-Dri solution (Novex).

Component	Composition	Volume added (sufficient for 4ml/2 gels)
Distilled water	-	2.4ml
Stacking buffer	0.5M Tris, 0.014M SDS, pH 6.8 with HCl	1ml
30% Acrylamide	Acrylamide/bis acrylamide 29:1	0.6ml
10% APS	0.44M	24µl
TEMED	-	8µl

Table 2-7: Composition of stacking gel for PAGE

2.3.3 Western Blotting

2.3.3.1 Transfer of Protein from Polyacrylamide Gel onto Nitrocellulose Membrane

To transfer proteins from SDS PAGE gels onto the surface of nitrocellulose membranes for analysis by Western blotting, the following materials were pre-soaked in transfer buffer (25mM tris, 192mM glycine, 20% methanol). These were placed in order, in a gel holder cassette: a fibre pad, 2 pieces of blotting paper, the polyacrylamide gel, a 4x9cm piece of Amersham Hybond C nitrocellulose, two more filter papers and the second fibre pad.

The cassette was placed in a miniprotein II Western blot apparatus (Bio-Rad), which was filled with transfer buffer. Transfer was then carried out at 95V for 75 minutes or overnight at 30V.

2.3.3.2 Detection of Protein with Antibodies

After the transfer, the nitrocellulose membrane was washed for 5 minutes in PBS containing 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate Sigma P-5927). Non-specific binding on the filter was prevented by addition of 3% Marvel skimmed milk in PBST for at least 30 minutes. The filter was then washed twice in PBST for 5 minutes before incubation with the primary antibody, diluted typically to 1:1000 concentration, in 3% skimmed milk in PBST for 45 minutes. The nitrocellulose was again washed twice in PBST then incubated with an appropriate secondary antibody, typically at 1:5000, for 45 minutes. Two more PBST washes were followed by a final 5 minute wash in PBS without tween. Substrate solution (30mg 4-chloro-1-napthol tablet (Sigma), 10ml methanol + 30µl H_2O_2, in 50 ml PBS, mixed together immediately before use) was added and incubated at RT until

bands had developed to sufficient brightness. The reaction was then stopped by removal of the substrate by washing the nitrocellulose in distilled water.

2.3.4 Detection of Low Concentration Proteins by Chemi-Luminescent Signalling

When attempting to detect protein expression in eukaryotic cells driven by the DNA constructs, it proved necessary to add the solution containing the protein sample directly onto the nitrocellulose membrane in a volume of no more than 10µl per sample. This allowed production of a concentrated spot of sample. Once the samples had dried, the membrane was blocked in a solution of 1% bovine serum albumin (BSA) in PBS for 60 minutes at RT. Antibodies were added and incubated and washes were carried out as described for the normal Western blotting technique except that lower concentrations of antibodies were used, 6 washes were carried out at each stage and 3% milk PBST was replaced in all cases with 1% BSA PBST.

To enhance the signal generated a chemiluminescent substrate was added to the blot. This substrate was prepared by combining equal volumes of the luminol/enhancer solution and the stable peroxide solution (Pierce), and was added to the membrane in a plastic cover for 5 minutes. To detect the released light, the developed blot was then placed against a piece of photographic film (Amersham) and exposed for 60 seconds before developing as normal.

2.3.5 Electron Microscopy of HBcAg Samples

Samples of commercial HBcAg (Biodesign International, R3B601) at a concentration of 1µg/ml were negatively stained and examined by transmission

electron microscopy with the technical assistance of the University of Glasgow Integrated Microscopy Facility.

2.4 Tissue Culture Protocols

All centrifugation steps carried out with eukaryotic cells were at 450g for 5 minutes unless otherwise stated. All incubations were at 37°C with 5%CO_2 in a humidified incubator.

2.4.1 Passage of COS-7 Cell Line

COS-7 cells are an African green monkey kidney epithelial cell line. This cell line expresses large quantities of recombinant antigens from transfected plasmid DNA.

Adherent cells were grown in complete DMEM media (cDMEM: DMEM; Gibco 41965-039, 10% FCS Harlan Serolabs 10108-165, L-glutamine Gibco 25030-032, penicillin-streptomycin Gibco 15070-022), in 25 or 75cm^2 vented tissue culture flasks (Costar). Cells were split when ~80% confluent, as follows. The media was poured off and discarded. The cells were washed in Hanks balanced salt solution (Sigma H9394) to remove any residual media or dead cells and then treated with 5ml of 10x trypsin EDTA solution (Gibco) made up in Hanks solution. The flask was incubated at 37°C for no more than 5 minutes after which the cells could be dislodged. The trypsin was inactivated by adding 45ml cDMEM and mixing gently. The cell suspension was then centrifuged and the cell pellet resuspended in cDMEM. To maintain the cell line, typically, 10% of this volume was added to a

new 75cm^2 flask, the final volume of which was made up to 20ml cDMEM and then re-incubated at 37°C, 5%CO_2.

2.4.2 Passage of Non-Adherent Cell Lines

P815/c and RBL5/c cells were used in CTL assays. Both cell lines were kindly provided by Reinhold Schirmbeck, Institute of Medical Microbiology and Immunology, University of Ulm.

Both cell lines had been transfected with HBcAg CD8$^+$ epitopes: P815, an H-2^d mastocytoma cell line expressed an H-2^d epitope, while the Rauscher virus-transformed T lymphoma line RBL5 expressed an H-2^b epitope (Kuhrober *et al.* 1996);(Kuhrober *et al.* 1997).

These lines were cultured in complete RPMI (cRPMI: RPMI 1640, Sigma R8055, 10%FCS, L-glutamine, penicillin/streptomycin, 0.05mM β-mercapto-ethanol) in 75cm^2 vented tissue culture flasks. Cells were passaged by taking typically 10% of the cell culture, adding these cells to a new flask and making it up to the previous volume with fresh cRPMI.

2.4.3 Lipofectamine Transfection of COS-7 Cells

COS-7 cells were seeded into a 24 well plate at 1-5x10^5 cells/well, in 1ml cDMEM per well. The plates were incubated for 24 hours at 37°C, and 5%CO_2. The cRPMI was removed from the wells and replaced, following a wash with 1ml Hanks solution, with 1ml cDMEM minus antibiotics.

DNA was added to lipofectamine at a ratio to ensure that for each 2µl of lipofectamine delivered per well, the complexes contained up to 1µg DNA. These

complexes were prepared by admixing 50µl of the DNA, resuspended in incomplete RPMI, with 50µl of lipofectamine solution. The mixture was then incubated for 20 minutes to allow DNA/lipofectamine complexes to form. To transfect the cells, the media above the cells was removed and replaced with 100µl of DNA/lipofectamine mixture per well. After 4 hours at 37°C, an additional 900µl of cDMEM was added to each well and the plates returned to the incubator for up to 72 hours.

To measure the level of protein produced from the DNA vaccine, cells were recovered from the wells using trypsin 72 hours after transfection. The cells were then lysed using high speed centrifugation (14krpm for 10 minutes) and the resultant material was concentrated at least 5 fold by allowing evaporation of the water. Samples were then assayed for the presence of the protein by dot-blot as described earlier.

2.4.4 *Salmonella* Invasion Assays

Adherent, confluent monolayers of J774 mouse macrophages were prepared in 24-well plates by seeding at $1x10^5$ cells/well 24 hours before starting an infection assay. To ensure antibiotics used in the media during routine passage of the cells did not affect bacterial viability, cells were washed with incomplete RPMI 1 hour prior to the start of the experiment.

10µl of *Salmonella* from an overnight culture were added per well (the precise number of bacteria was subsequently determined by preparation of viable counts), giving an initial dose of approximately $1x10^7$ bacteria per well. To bring the bacteria into close proximity to the cell monolayer, the plates were centrifuged at 450g for 5 minutes. Bacteria were then allowed to interact with the cells for 2 hours before

free bacteria were removed by washing with sterile PBS. Extracellular bacteria were killed by the addition of incomplete RPMI containing 100μg/ml gentamycin for a minimum of 1 hour. At various time points following this treatment, monolayers were washed and cells lysed with 1% triton X 100. Serial dilutions of the contents of each well were prepared and plated out to quantify the number of viable intracellular bacteria. These are expressed as colony forming units (CFU)/ml.

2.5 *In Vitro* Immunoassays

2.5.1 Enzyme Linked ImmunoSorbent Assays (ELISAs)

2.5.1.1 ELISA to Determine Serum Antibody Levels

Sera were prepared from blood samples taken from mice by hypodermic needle from the tail vein or from cardiac bleeds. Serum was routinely separated from the red blood cell component by centrifugation at 14,000rpm for 5 minutes.

96 well ELISA plates (Corning Costar 96 well ELISA plates 3590) were coated with the appropriate antigen (e.g. HBcAg; Biodesign international R3B601, at 0.5μg/ml) in sodium carbonate coating buffer (0.1M Na_2HCO_3 in distilled water at pH 8.2) in a final volume of 50μl/well. The plates were then incubated for a minimum of 2 hours at 37°C before being washed with PBST. Wells were then blocked with 150μl/well 10% newborn calf serum (NCS; Gibco 16010-159) in PBS and incubated for another hour at 37°C. Plates were washed as before and dilutions of mouse sera prepared in 10% newborn calf sera. Samples were tested from 1:50 for sera (neat for mucosal washes) in the top well and serially diluted by 5 fold increments. This gave a range of dilutions from 1/50 to ~1/3.9x10^6. In addition, on each plate, a standard positive and negative control sera was added at the same

dilutions. This allowed direct comparison of data from different samples tested on the same day or samples tested on different days.

Following addition of the sera, plates were returned to the incubator for a minimum of 1 hour. Following careful washing of the plates (using PBST) 50µl detecting antibody was added (e.g. rabbit anti-mouse HRP conjugated antibody; Dako PO260, diluted to 1:1000 in 10% NCS/PBST) to each well. The plates were again incubated for a minimum of 1 hour before being washed and developed using 50µl of substrate solution (0.05M citric acid, 0.125M Na_2HPO_4, 0.006% H_2O_2, 1 tablet O-phenyldiamine-dichloride (OPD; Sigma P-8287) per 50ml). Once the maximum colour change had been completed (5-15 minutes), the reaction was stopped by the addition of 50µl of 3M HCl per well, which inactivates the enzyme. The colour change in the wells was then measured and the absorbance recorded.

Plates were read on an Elx 808 Ultramicroplate reader (Bio-tek instruments inc.) at a wavelength of 490nm. The data were analysed using the program Kineticalc 4 (Bio-tek instruments) and Microsoft Excel. Endpoint titres were calculated as the reciprocal of the highest dilution, which gave an absorbance of 0.3U above the background absorbance observed in the negative control wells. All titres were standardised against the same positive control sera.

2.5.1.2 ELISA to Measure Immunoglobulin A at Mucosal Surfaces

These ELISAs were carried out in a similar manner to those for serum antibody with the following exceptions. Firstly, mucosal washes were added neat to the top row of the ELISA plate (and then diluted using 5 fold serial dilutions down the plate, as with serum samples). Secondly, the detection antibody used in these assays was an α-chain specific goat anti-mouse biotinylated antibody (Sigma B-

2766) diluted at 1:500. Binding of this antibody was subsequently detected using streptavidin-horse radish peroxidase conjugate (Dako P0397) added to the wells at a concentration of 1:1000.

2.5.1.3 ELISA to Determine Lipopolysaccharide-Specific Antibody Levels

The coating antigen used in these ELISAs was *S. typhimurium* LPS (Sigma L-6511). This was prepared by dissolving 500µg of LPS in 1ml 5x Reggiardo's buffer (0.25M glycine, 0.5M NaCl, 0.005M EDTA, 0.25M NaF) and then adding 4ml distilled water, providing antigen sufficient to coat a single ELISA plate at 50µl/well. The remainder of the assay for detection of LPS specific antibodies is otherwise identical to that described above.

2.5.2 Preparation and Culture of Murine Lymphocytes

Mice were killed by cardiac bleed under terminal anaesthesia followed by cranial dislocation. Spleens and lymph nodes were then recovered and stored in ice-cold RPMI media until required. To generate single cell suspensions, tissues were placed in sterile petri dishes and were crushed using the blunt end of a sterile disposable syringe with the rubber cap removed. Cells were then recovered in approximately 5ml of sterile RPMI. To purify single cell suspensions from spleen tissues, the crushed tissue was additionally subject to a brief centrifugation step (to remove large masses of debris and connective tissue) and the supernatants were decanted into fresh tubes. The cells were then pelleted by centrifugation and erythrocytes removed by the addition of 1ml ACK red blood cell lysis buffer (160mM NH_3Cl, 100mM $KHCO_3$, 100mM EDTA) for 2 minutes. This reaction was then stopped by addition of 5 volumes of RPMI and unlysed white cells

recovered by centrifugation at 200g for 10 minutes. In contrast, cells from the lymph nodes were passed through sterile 100µm nylon mesh, ('nytex' Cadlish) to remove any remaining debris and adipose tissue, and then cells pelleted by centrifugation.

Once single cell suspensions were prepared they were counted using a haemocytometer before being used in further applications.

2.5.3 Magnetic Cell Separation (MACS)

In order to isolate a particular cell type from a population (either to be used as the sole cell type in an assay or to be specifically excluded from an assay) cells were sorted using a magnetic cell separation system. To label the cells with the appropriate magnetically tagged antibody, cells were initially resuspended in MACS buffer (0.5%BSA, 2mM EDTA in sterile PBS) at $1x10^7$ cells per 90µl buffer. 10µl of MACS beads bound to antibody of the appropriate specificity (Miltenyi Biotec) were added to every 10^7 cells. The cells and beads were incubated together at 4°C for 15 minutes. The cells were then washed to remove any unbound antibody and the cells finally resuspended in 1ml MACS buffer.

A washed magnetic column (LS 130-041-306) was then placed in the MACS magnet and the labelled cell suspension added. A collection tube was placed under the column and the column washed through with a minimum volume of 5ml of MACS buffer. This ensured the removal of any unbound cells.

For positive selection, e.g. T cell selection, the flow through was discarded and the T cells collected by removing the column from the magnet, adding 5ml more MACS buffer and expelling the cells into a new collection tube.

For positive deletion, e.g. B cell depletion, the flow through was retained and the column, containing bound B cells, was discarded.

The selected cells were counted, washed and resuspended in cRPMI for further applications.

2.5.4 Preparation of APCs for Proliferation Assays

2.5.4.1 Irradiation of APCs

Spleen cells were separated into a single cell-suspension and irradiated with 2500 rads from a Cobalt-60 source.

2.5.4.2 Mitomycin C Treatment of APCs

Cells were treated with 50µg mitomycin C (Sigma) per 10^7 APCs and incubated for 75 minutes. The cells were washed in a large volume of culture medium four times to remove all traces of mitomycin C prior to use in cultures.

2.5.4.3 Culture of Murine DCs

DCs were prepared from culture of bone marrow cells prior to the proliferation assay (Lutz *et al.* 1999). The femur was removed from recently killed mice of the same strain to be examined in the proliferation assay. The marrow was removed by washing with sterile PBS and a single cell suspension prepared. The cells were incubated in 75ml flasks in 20ml of complete RPMI containing 2ml GM-CSF solution. The GM-CSF was a kind gift of Dr. J. Brewer (University of Glasgow) and was obtained from transgenic cell line supernatants (Zal *et al.* 1994). Cells were incubated and fed with fresh media containing GM-CSF (9ml cRPMI

plus 1ml GM-CSF) 3 and 6 days later. On the 7th day cells were harvested and used as mature APCs in the proliferation assay.

2.5.5 Proliferation of Restimulated Lymphocytes Measured by Tritiated Thymidine Incorporation

Following preparation of a single cell suspension of the appropriate lymphocyte population, cells were added to the wells of a round-bottomed 96 well plate (Corning/Costar L6950) at a concentration of 10^5 cells/well in a final volume of 100µl. Cells from each experimental group of mice were added to 12 wells, to give four sets of triplicate wells, which were then tested using 3 different concentrations of restimulating antigen. Following addition of antigen, cells were incubated for 72 hours and then pulsed with 1µCi ^{3}H-thymidine (Amersham) per well. The plates were incubated for a further 12-16 hours before harvesting.

Cells were harvested onto a glass fibre filtermat using an automated MACH III cell Harvester (TomTec, USA). These mats were then dried before being placed in a plastic sample bag with 4ml scintillant and analysed using a Trilux MicroBetacounter (PerkinElmer). Measured counts per minute (cpm) were generated for each well and mean data and standard deviations calculated using Microsoft Excel.

2.5.6 Cytotoxic T lymphocyte Killing Assay

2.5.6.1 5 Day Restimulation

The appropriate target cells (i.e. RBL5/c or P815/c, see tissue culture protocols) were grown up in bulk prior to recovery of CTLs from immunised

animals. The target cells were centrifuged and resuspended in an appropriate volume of cRPMI. To prevent proliferation of these cells, 50µg mitomycin C was added per 10^7 cells. The cells were incubated for 75 minutes (37°C, 5%CO_2) and then washed 4 times in cRPMI.

Single cell suspensions of spleen cells were added to the target cells in 25cm^2 tissue culture flasks at a final concentration of $1.5x10^6$ target cells and $3x10^7$splenocytes. To ensure the generation of sufficient effector cells, 4 or 5 flasks were prepared per experimental group and amplification was allowed to proceed by incubation of the two populations for 5 days at 37°C, 5%CO_2.

2.5.6.2 ^{51}Chromium Release Assay

To label the target cells, $2x10^7$ fresh target cells and the same number of control cells, were incubated separately with 5MBq sodium chromate (^{51}Cr) each, for 120 minutes.

Restimulated cells from 5-day incubation were washed, counted and resuspended at $1x10^8$/ml. These cells were plated out in four rows of 6 wells containing two-fold dilutions from 10^7 cells/well to $1.25x10^6$ cells/well. To half of these wells 10^5 target cells were added whilst the equivalent numbers of control cells were added to the remaining wells. This gives effector : target cell ratios ranging from 100:1 to 12.5:1. As controls, six wells were prepared which contained either no antigen or effector cells. To allow estimation of the uptake of Cr^{51} by the cells, three of these wells were treated with 10% Triton-X100. This completely lyses the cells and releases the radioactivity into the surrounding media.

The target and effector cells were allowed to interact for 4 hours before 25µl of media from above the cells was carefully removed and transferred to a flexiplate

where it was mixed with 150µl scintillation fluid. The amount of radioactivity present in each well was analysed using a Trilux Microbeta plate reader. Percentage lysis was calculated as:

2.5.7 Cytotoxic T lymphocyte IFN-γ Secretion Assay (ELISPOT)

T cells were isolated using the pan T cell MACS beads as described previously and restimulated with target cells for 5 days.

To allow detection of IFN-γ release, a 96 well multiscreen plate (Millipore) was coated with 50µl of anti mouse IFN-γ antibody (Pharmingen, UK. 5µg/ml) overnight at 4°C. The plates were the washed and blocked for 1 hour in cRPMI before addition of the restimulated cells. The isolated T cells were added in duplicate wells to APCs that had previously been treated with antigen or $CD8^+$ peptide and the volume of media in each well made up to 200µl using cRPMI. The cells were then co-incubated for 20 hours before removal by vigorous washing. IFN-γ released and captured was then detected using a biotinylated anti-IFN-γ antibody (Pharmingen, UK; 1µg/ml), which was added in cRPMI for 1 hour at 37°C.

To detect the bound biotinylated antibody the plates were treated with 100µl extravadin alkaline phosphatase at 1:1000 of a 1.5mg/ml solution and incubated for 1 hour. The plate was then washed and 50µl of insoluble developing solution added (30mg tablet of BCIP/NBT in 10ml distilled water) to each well. Spots then developed on the base of the wells and were counted using a low

powered microscope at 32x magnification. Mean counts of triplicate wells were calculated.

Finally, the number of antigen specific IFN-γ producing cells was determined by subtracting the number of spots observed in wells containing unstimulated cells from those which had been stimulated with peptide or protein.

2.6 *In Vivo* Protocols – Vaccinations and Acquisition of Samples

2.6.1 Intra-muscular (i.m.) Protein and DNA Vaccination

Mice were immunised in the hind quadricep by hypodermic injection. Vaccine DNA or protein was delivered in a volume of 100µl sterile, LPS-free PBS, 50µl to each leg.

2.6.2 Gene Gun DNA Vaccination

24 hours prior to vaccination the region of the skin to be immunised was shaved. When vaccinating, the gene gun was positioned against the mouse skin, separated by a 2.5cm spacer, and the coated gold particles driven into the skin using helium pressurised to 40psi.

2.6.3 Intra-Gastric Immunisation with *Salmonella*

S. typhimurium BRD 509 were grown statically overnight at 37°C. They were harvested by centrifugation in a Sorval SS34 rotor, washed in LPS-free PBS and

resuspended to $5x10^{10}$ bacteria/ml. Bacteria were delivered to anaesthetised mice using a gavage needle in a final volume of 200µl.

2.6.4 Collection of Blood Samples

Sample bleeds were taken from mice at stages throughout experiments. Hypodermic needles were used to extract approximately 50µl of blood from the tail vein of restrained mice. Samples were allowed to clot for approximately 3 hours at room temperature or overnight at 4°C. The samples were then centrifuged at 10,000g for 5 minutes and the supernatant collected. This serum was then further cleaned by centrifugation for a further 10 minutes and removal of the supernatant to a fresh tube. Samples were stored at -80°C.

If serum samples were required at the end of the experiment, mice were terminally anaesthetised with halothane and exsanguinated by cardiac puncture.

2.6.5 Collection of Mucosal Washes

2.6.5.1 Nasal and Pulmonary Washes

Following exsanguination, the trachea of the mouse was exposed and punctured. A sharpened, fine tipped pastette was used to infiltrate the lungs with up to 1.5ml of 0.1% BSA in PBS. Following 3-4 flushings, as much of the fluid as possible was recovered and used in subsequent analysis.

Nasal washes were obtained by separating the head of the mouse from the body and removing the lower jaw. A fine-tipped pastette was used to introduce 1ml of 0.1% BSA in PBS via the Eustachian tube through the nasal passages of the

mouse. Samples were collected as this wash emerged from the nostrils of the animal. Samples were stored at -20°C prior to analysis.

2.6.5.2 Intestinal Washes

The upper intestine was dissected from the body and separated into ~5cm sections. Digested material was gently removed from each section and the tissue was washed through twice with 1.5ml of PBS containing 0.1% BSA and 1mM Phenyl-methane-sulphonyl-fluoride (PMSF). Samples were stored at -20°C prior to analysis.

2.7 Statistics

Data were analysed with Student's two-sample t test using Microsoft Excel. Differences in variation were calculated and taken into account when deciding whether to use the test for equal or unequal variance. The p values shown in the text indicate the probability of the two samples analysed being different. Values over 0.95 were considered significant.

3 RESULTS – VACCINE DEVELOPMENT

3.1 Chapter Aims

The aim of work described in this chapter was to characterise the different vaccine formulations used in the course of this project. This includes their preparation prior to their testing *in vivo*.

3.2 Introduction

Investigations carried out during the course of this project were based on the presentation of one antigen, HBcAg, to the immune system using different vaccination techniques. Whilst some of these vaccines were prepared from commercially available material, others were synthesised in the laboratory. Following production, each vaccine type required characterisation *in vitro*, to demonstrate the suitability of the formulation for vaccine delivery.

3.3 Routes and Types of Vaccine

Although based on the delivery of the same antigen, this study aimed to consider the type of immune response generated when the antigen is delivered in three very different forms:

- as a purified recombinant protein,
- encoded on purified plasmid DNA vaccine
- expressed from a plasmid DNA carried by an attenuated strain of *Salmonella*.

In addition, the antigens were delivered either parenterally or mucosally. The different methods of vaccination used are described in Table 3-1.

3.4 Vaccine Formulations

3.4.1 Purified HBcAg Vaccinations

Purified HBcAg obtained from a commercial source was used for both parenteral and mucosal vaccinations. This recombinant protein, obtained from Biodesign International, (product number R3B601), was expressed in *P. pastoris* from an expression vector encoding the entire 183 amino acids of the antigen. The resultant 21kDa protein was described as having purity of >95% and required only dilution in PBS prior to vaccination. However, to ensure the protein was as described in the certificate of analysis, it was additionally analysed prior to use by PAGE, Western blot and by electron microscopy (conducted as described in Materials and Methods sections 2.3.2, 2.3.3 and 2.3.5). Figure 3-1A shows that under denaturing conditions, the protein can be seen as a monomer of approximately 21KDa. Figure 3-1B shows that by Western blot this protein reacts strongly with polyclonal anti-HBcAg sera. In addition, the same blot shows two additional bands of approximately 42 and 64KDa. It is believed that these bands represent dimers and trimers of the protein. This indicates the strength of binding between monomers, which treatment with β-mercaptoethanol failed to reduce entirely to the monomeric form.

Route of Vaccination		Vaccine Formulation
Parenteral	Intra-muscular	DNA expressing HBcAg
	Gene gun	DNA expressing HBcAg
	Intra-muscular	Purified HBcAg protein
Mucosal	Intra-nasal	Purified HBcAg protein
	Intra-gastric	Attenuated *Salmonella* expressing HBcAg

Table 3-1: Vaccination strategies examined throughout this project

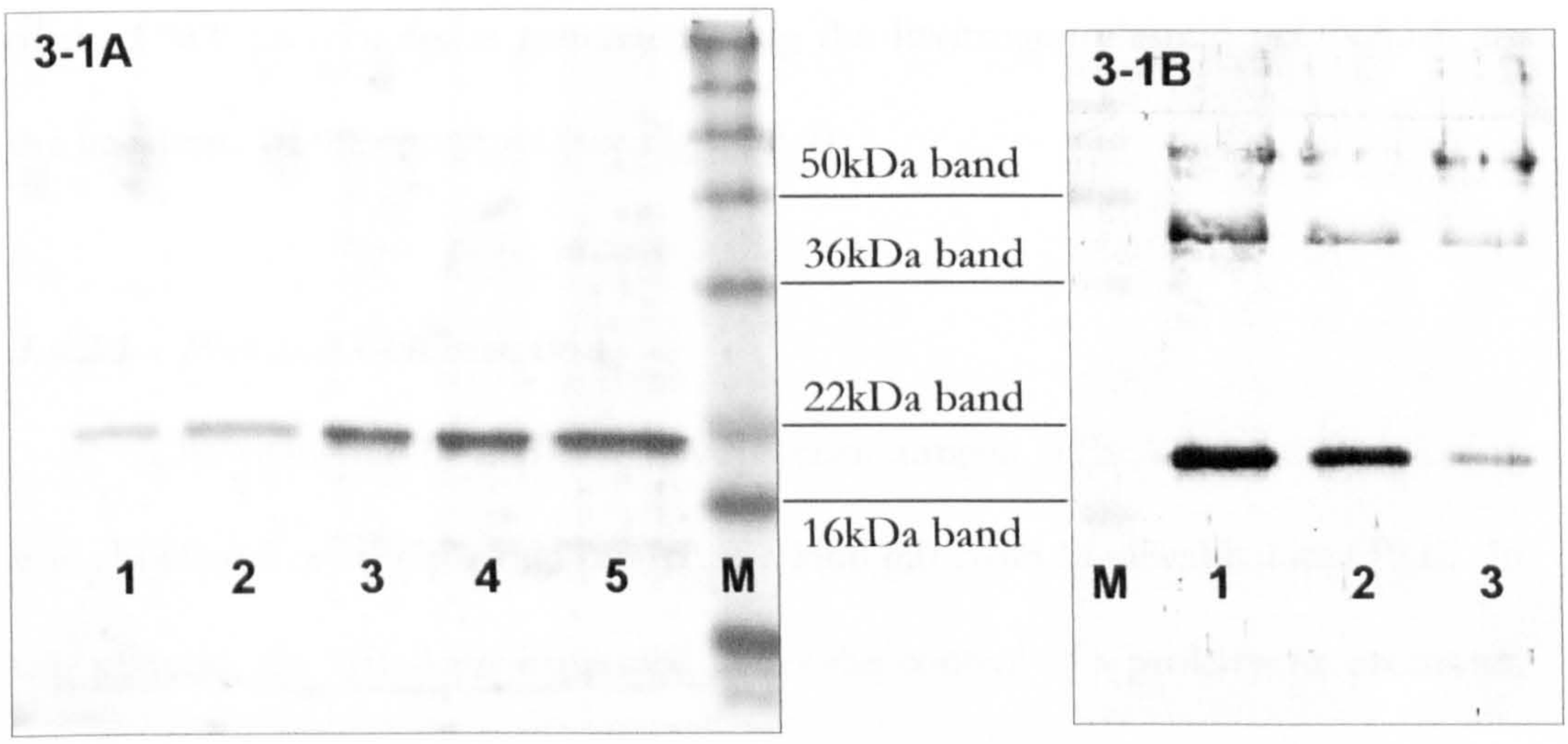


Figure 3-1: Characterisation of commercial HBcAg under denaturing conditions by SDS PAGE and Western blot analysis

Figure 3-1A shows different quantities of HBcAg visualised by Coomassie blue stain. Lanes 1 – 5 contain 0.25µg, 0.5µg, 0.75µg, 1.0µg and 1.5µg of protein. Lane M contains standard markers.
Figure 3-1B shows the binding of anti-HBcAg antibody to the protein. Lanes 1 – 3 contain 1.5µg, 1.0µg and 0.5µg of protein.

To confirm that the purified material was particulate in nature at the time of vaccination, the material was also analysed by transmission electron microscopy. A negatively stained sample of the HBcAg is shown in Figure 3-2. This clearly shows assembled core particles of the expected size; approximately 350 Angstrom (see Introduction section 1.6.3, Structure and Immunogenicity of the Core Antigen).

3.4.2 Generation of a Naked DNA Vaccine Encoding HBcAg

A DNA vaccine containing the cloned HBcAg sequence under the control of the CMV promoter was generated using the Invitrogen plasmid pcDNA3.1$^+$ as the backbone of the construct (see Figure 2-1).

3.4.2.1 Plasmid Construction

The sequence of hepatitis B virus core antigen (HBcAg) used for cloning was obtained from the plasmid p*trc*/core, a kind gift from MedevaPharama PLC. In this plasmid, the HBcAg is expressed under the control of a prokaryotic promoter, which allows the production of this protein in bacteria.

In order to create a plasmid that could be used in DNA vaccination, the sequence was cloned into the plasmid pcDNA3.1 (Invitrogen P790-20) using the strategy outlined in Figure 3-3. In brief, primers were designed (BJS 1 and BJS 2, see Table 2-3) that would amplify the HBcAg DNA and introduce restriction enzyme cutting sites close to the 5' (*Hin*d III) and the 3' (*Bam*H I) ends of the PCR product. After removal of the enzyme and buffer salts using the Qiagen PCR purification kit, the PCR product was subjected to digestion with *Hin*d III and *Bam*H I (see Materials and Methods section 2.2.5). The digested DNA was then run on a 0.8% agarose gel and the DNA fragment cut and purified from the gel (Qiagen

gel extraction kit). Simultaneously, the vector pcDNA3.1 was digested with the same enzymes and purified using the same method. The purified HBcAg sequence and pcDNA3.1 fragments were combined and ligated to give a new plasmid, designated pcDNA3.1/core, which was transformed into competent *E. coli* XL10. Ampicillin resistant colonies were then screened by PCR for the presence of the HBcAg sequence using specific primers (BJS4 and 5), see Figure 3-4. Insertion of the HBcAg sequence was additionally confirmed by digest of plasmid isolated from PCR positive colonies. Figure 3-5 shows single and double digests of both the original plasmid (pcDNA3.1) and the cloned plasmid pcDNA3.1/core. Whilst it is difficult to observe a difference of 500bp between the plasmids following linearisation with a single enzyme (lanes 2 and 5), a fragment of approximately 550bp is visible when pcDNA3.1/core is digested with both enzymes (lane 6). This corresponds to the size of the HBcAg gene. This band is absent when pcDNA3.1 is similarly digested (lane 3). To verify the sequence of the cloned DNA, the isolated plasmid was sequenced. The data in Table 3-2 confirms the expected sequence of the inserted DNA.

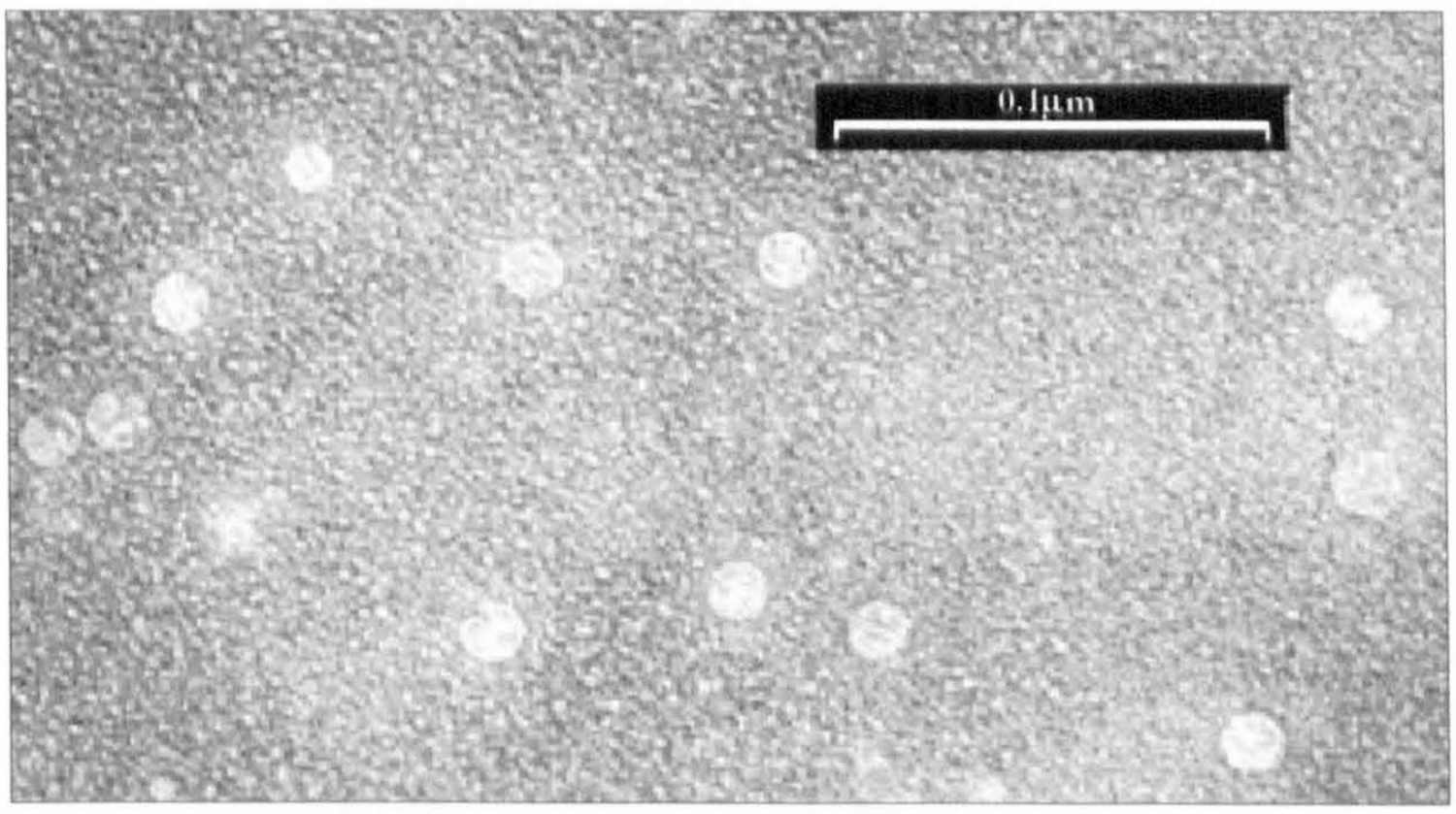


Figure 3-2: Electron micrograph showing assembled HBcAg particles

Transmission electron microscopy of a negatively stained sample of commercially obtained HBcAg expressed in *P. pastoris*, viewed at 80,000x magnification.

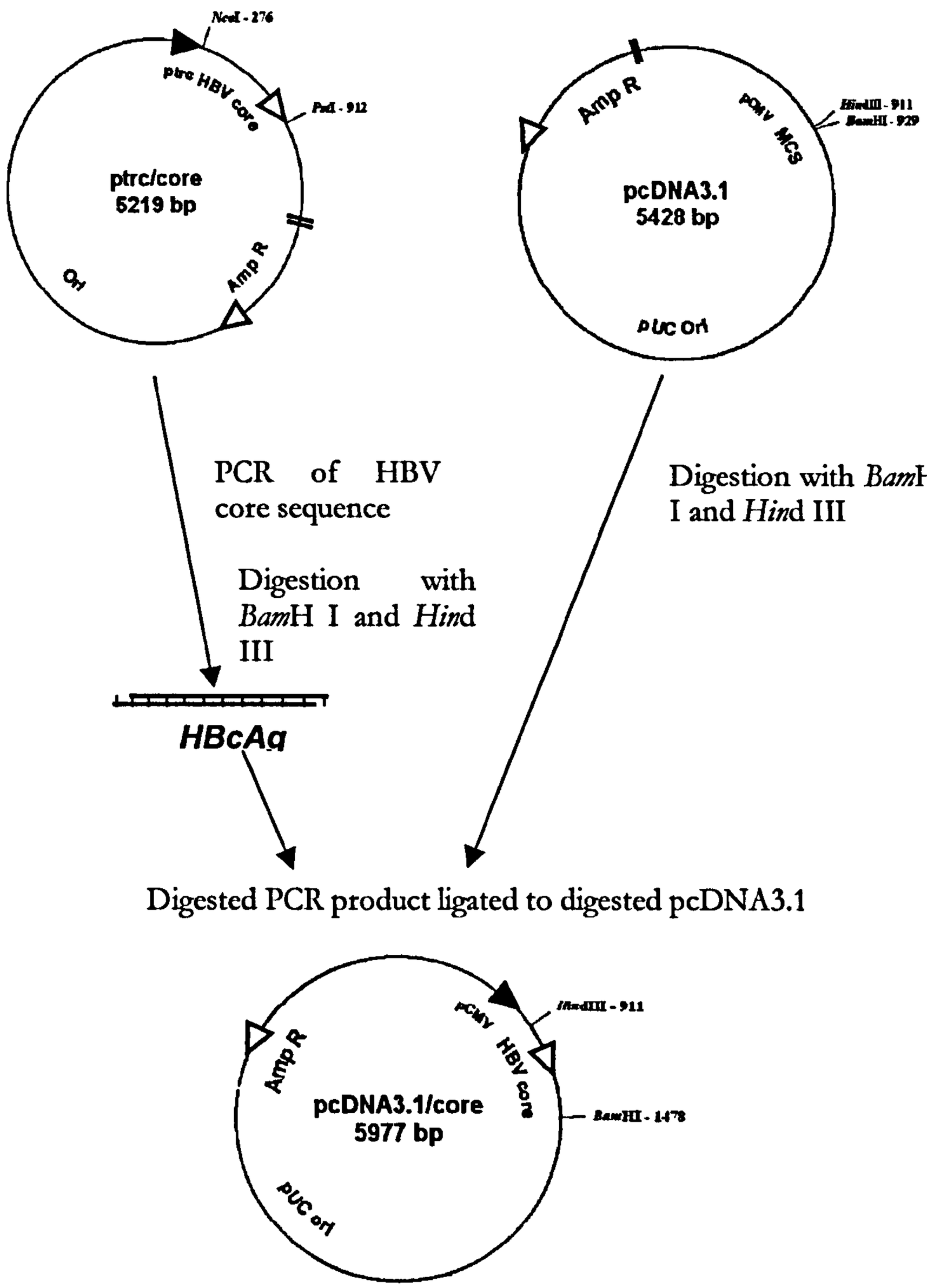


Figure 3-3: Schematic showing the cloning steps involved in the generation of pcDNA3.1/core

The HBV core sequence was amplified from ptrc/core by PCR with modified primers encoding the restriction enzyme recognition sites *Hin*d III and *Bam*H I flanking the HBcAg reading frame. The PCR product was digested with *Hin*d III and *Bam*H I and ligated to the similarly digested plasmid pcDNA3.1. The resulting construct, designated pcDNA3.1/core, expresses the HBV core sequence under the control of a eukaryotic promoter.

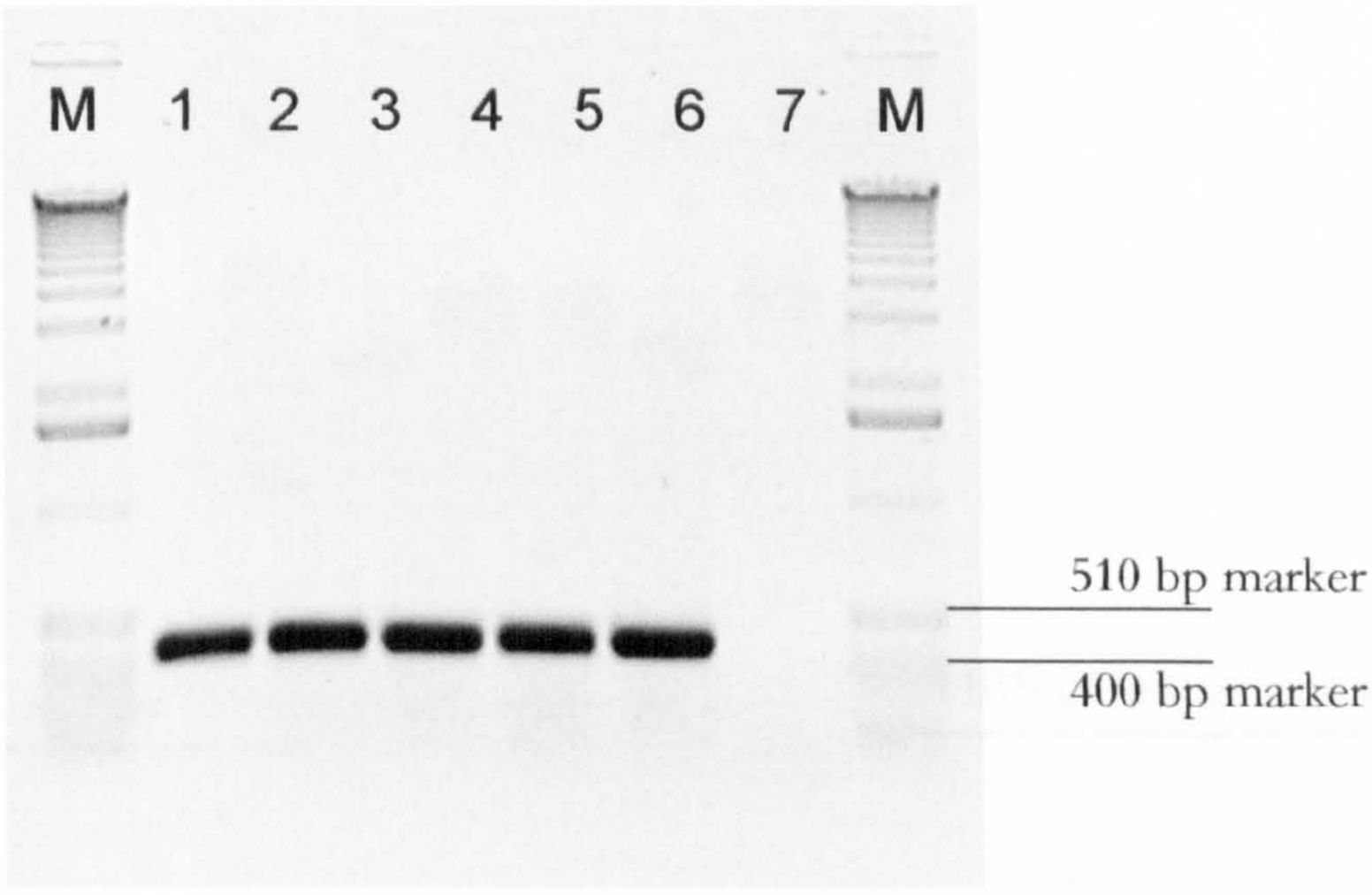


Figure 3-4: Screening of *E. coli* XL-10 bacteria for the presence of HBcAg DNA following transformation with pcDNA3.1/core

Following the transformation of *E. coli* with the pcDNA3.1/core plasmid, the presence of the core sequence was confirmed by PCR using primers internal to the HBV core sequence. These primers were expected to amplify a band of approximately 450bp, which was observed in all ampicillin resistant colonies screened (lanes 1-6). No such band was present when PCR was conducted using untransformed, ampicillin sensitive *E. coli* (lane 7).

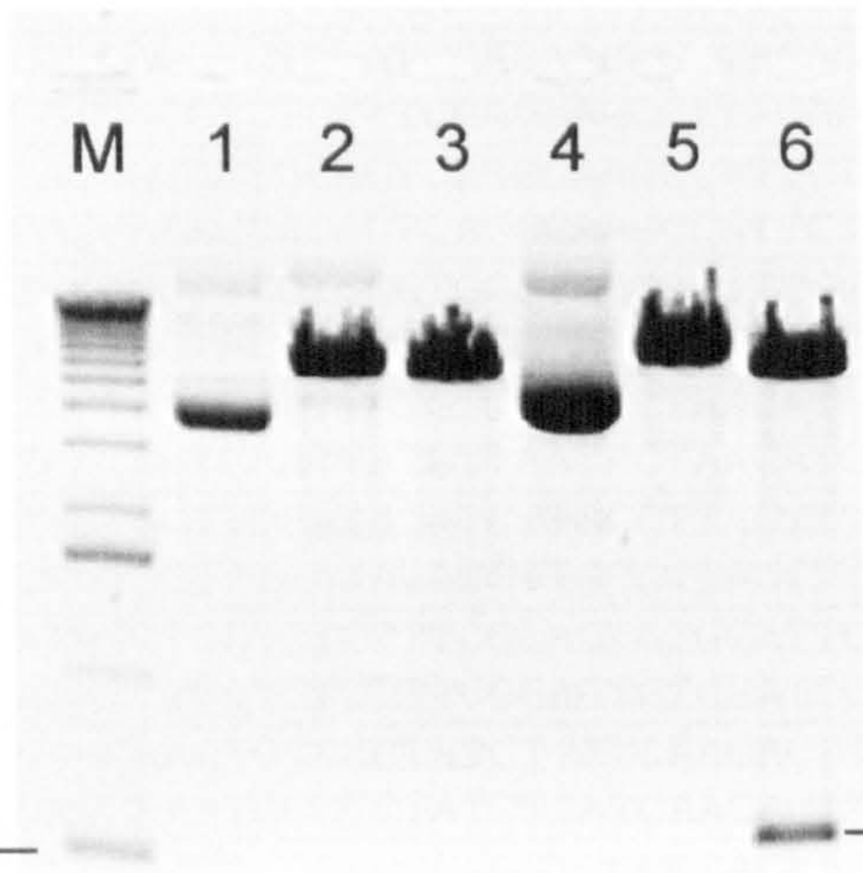


Figure 3-5: Confirmation of insertion of HBcAg sequence by restriction digest of pcDNA3.1 and pcDNA3.1/core plasmids

Lanes 1, 2 and 3 contain undigested, singly digested (*Hin*d III) and doubly digested (*Bam*H I) pcDNA3.1 respectively. Lanes 4 – 6 contain the same digests of pcDNA3.1/core.

Position	Sequence: top row pcDNA3.1 core bottom row ptrc/core
1	ATGGACATTGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTC ATGGACATTGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTC
51	GTTTTTGCCTTCTGACTTCTTTCCTTCCGTCAGAGATCTTCTAGACACCG GTTTTTGCCTTCTGACTTCTTTCCTTCCGTCAGAGATCTTCTAGACACCG
101	CCTCAGCTCTGTATCGAGAAGCCTTAGAGTCTCCTGAGCATTGCTCACCT CCTCAGCTCTGTATCGAGAAGCCTTAGAGTCTCCTGAGCATTGCTCACCT
151	CACCATACTGCACTCAGGCAAGCCATTCTCTGCTGGGGGGAATTGATGAC CACCATACTGCACTCAGGCAAGCCATTCTCTGCTGGGGGGAATTGATGAC
201	TCTAGCTACCTGGGTGGGTAATAATTTGGAAGATCCAGCTAGCAGGGATC TCTAGCTACCTGGGTGGGTAATAATTTGGAAGATCCAGCTAGCAGGGATC
251	TAGTAGTCAATTATGTTAATACTAACATGGGTTTAAAGATCAGGCAACTA TAGTAGTCAATTATGTTAATACTAACATGGGTTTAAAGATCAGGCAACTA
301	TTGTGGTTTCATATATCTTGCCTTACTTTTGGAAGAGAGACTGTACTTGA TTGTGGTTTCATATATCTTGCCTTACTTTTGGAAGAGAGACTGTACTTGA
351	ATATTTGGTCTCTTTCGGAGTGTGGATTCGCACTCCTCCAGCCTATAGAC ATATTTGGTCTCTTTCGGAGTGTGGATTCGCACTCCTCCAGCCTATAGAC
401	CACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGA CACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGA
451	CGACGGGGCCGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAG CGACGGGGCCGAGGCAGGTCCCCTAGAAGAGAACTCCCTCGCCTCGCAAG
501	ACGCAGATCTCAATCGCCGCGTCGCAGAAGATCTCAATCTAGGGAATCTC ACGCAGATCTCAATCGCCGCGTCGCAGAAGATCTCAATCTAGGGAATCTC
551	AATGTTAG AATGTTAG

Table 3-2: Sequence analysis of cloned HBcAg in pcDNA3.1/core

This table shows a comparison of the cloned sequence with that of the known sequence from the p*trc*/core plasmid. The sequence shown in the top row is that of pcDNA3.1/core and that in the bottom row is p*trc*/core. The sequences are identical.

3.4.2.2 Expression of HBcAg from pcDNA3.1/core in Eukaryotic Cells

As expression of the core antigen in eukaryotic cells is a prerequisite for the vaccine to function, it was necessary to determine whether the newly synthesised pCDNA3.1/core plasmid was able to express antigen *in vitro*. This was demonstrated by transformation of COS-7 cells with pcDNA3.1/core using lipofectamine (see Materials and Methods section 2.4.3). Initially, cellular samples were subject to standard PAGE gel and Western blot analysis. However, these experiments failed to reveal any expression of the antigen. To improve the sensitivity of the assay, lysed cellular debris was dotted directly onto nitrocellulose membranes and a chemiluminescent secondary antibody used in the staining procedure (section 2.3.4). Using this method, the sensitivity could be improved from a lower limit of approximately 500ng using standard Western blot to between 1-10ng using the dotblot. Comparison with samples of the commercial HBcAg showed that cells transfected with pcDNA3.1/core were able to express around 5ng of protein per 10^4 transfected COS-7 cells (Figure 3-6).

Confirmation that this plasmid could direct the expression of antigen in eukaryotic cells suggested that the DNA generated could function as an effective DNA vaccine. Immune responses to DNA vaccination via two routes of immunisation were to be considered; i.m. and by particle bombardment with a gene gun.

3.4.2.3 Large Scale Preparation of DNA

Large quantities of pcDNA3.1/core were required for use in vaccination experiments. Following confirmation of eukaryotic expression, a large-scale preparation of the plasmid was undertaken. Using a Qiagen Megaprep Kit plasmid was isolated and then characterised by digestion with *Hin*d III and *Bam*H I to

confirm the presence of the core sequence. The DNA isolated quantified by UV spectrophotometry. Table 3-3 shows typical values recorded for the purity and concentration of the DNA sample by UV spectrophotometry. From a culture of 500ml, approximately 1-2mg of DNA was generated. This procedure was carried out on several occasions and each batch was characterised as described above.

3.4.2.4 Measurement of LPS Contamination of DNA

To determine the level of LPS present in the purified plasmid pcDNA3.1/core, LAL assays was performed on each batch of DNA prepared (see Materials and Methods section 2.2.13). Results of these tests showed that the plasmid produced was clean and uncontaminated (less than 0.1IU/100ug of DNA).

3.4.2.5 Preparation of Gene Gun Cartridges

In brief, purified DNA was bound to 1μm-diameter gold particles, which were then attached to the inside of a hollow plastic tube (external diameter approximately 2mm) using a special rotating apparatus to ensure an even coating of particles. Following attachment, the tubing was cut into 8mm-long sections, which could then be used as cartridges in the gene gun. The method is more fully described in Materials and Methods section 2.2.1.1.

The DNA attached to the cartridges was quantified by soaking the prepared cartridges in Tris/HCl overnight. The resultant supernatant was analysed by UV spectrophotometry. Typical results of this analysis are shown in Table 3-4 and confirm that approximately 1μg of each vector was bound per cartridge.

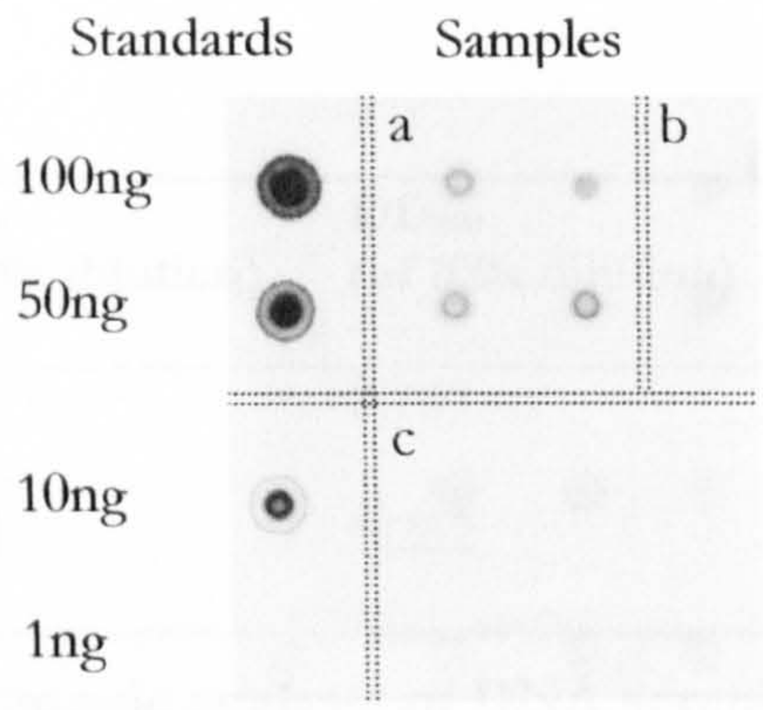


Figure 3-6: Expression of HBcAg in COS 7 cells

Expression of antigen in cells transfected with either pcDNA3.1/core (sample a), pcDNA3.1 (sample b) or untransformed cells (sample c). Expression detected by polyclonal mouse anti-HBcAg and rabbit anti-mouse HRP detected with chemi-luminescent substrate. Each sample represents ~10^4 lysed cells. The standard represents different quantities of commercial HBcAg dotted onto the membrane.

Sample	OD_{260} (of 100x dilution)	OD_{280} (of 100x dilution)	$OD_{260/280}$	Concentration of neat sample (mg/ml)
pcDNA3.1	0.354	0.187	1.893	1.77
pcDNA3.1/core	0.468	0.252	1.857	2.34

Table 3-3: Spectrophotometric analysis of DNA samples prepared by Qiagen Megaprep

Two typical examples of calculated concentrations of DNA purified from a 500ml overnight culture of bacteria are given here. Values were calculated using the following formula:

$$\text{Concentration (ng)} = 50 \times OD_{260}$$

The level of protein contamination of the sample is calculated by: $OD_{260} \div OD_{280}$. Protein contamination of the sample leads to significantly reduced values and renders the concentration calculation invalid. A value of 1.8 in a DNA sample is considered to be uncontaminated (Sambrook *et al.* 1989). This shows the DNA produced was of high quality.

Sample	Test cartridge number	OD_{260}	OD_{280}	DNA recovered (μg)	Group mean (μg), standard deviation
pcDNA3.1/core	1	0.206	0.161	1.03	1.102, 0.174
	2	0.297	0.126	0.985	
	3	0.185	0.126	0.925	
	4	0.266	0.171	1.33	
	5	0.248	0.181	1.24	
pcDNA3.1	1	0.264	0.192	1.32	0.975, 0.179
	2	0.222	0.174	1.11	
	3	0.193	0.139	0.965	
	4	0.220	0.166	1.1	
	5	0.145	0.131	0.725	

Table 3-4: Measurement of DNA from gene gun cartridges

DNA was recovered from cartridges by overnight treatment with Tris/HCl. DNA recovered was calculated as $[OD_{260}]$x50 = [DNA concentration]μg/ml. Samples were 100μl volume.

3.4.2.6 Attenuated Salmonella Vaccine Expressing HBcAg

The *S. typhimurium* strain used in these studies contained two attenuating deletions in the *aroA* and *aroD* genes (strain designated as BRD509). Expression of HBcAg was driven from the plasmid pGA-1, which encodes the protein under the control of an anaerobically induced promoter (*nir*B). As pGA-1 was provided in the first instance in *E. coli*, the first part of this work involved the transfer of the plasmid into the *Salmonella* strain.

3.4.2.7 Generation of the Salmonella typhimurium Vaccine Strain

Two considerations rendered the transformation strategy somewhat complex. The first involved the need to avoid degradation of the plasmid by the *Salmonella* due to inappropriate methylation. To achieve this, the plasmid was initially transformed into an intermediate strain; *S. typhimurium* LB5010 (Bullas and Ryu 1983), a *rec*A$^-$ strain that is unable to degrade the plasmid, but is able to methylate progeny plasmids appropriately for *Salmonella*. This has been described previously as a successful method for the production of *Salmonella* vaccine strains (Londono *et al.* 1996). This transformation was achieved by heat-shock of *S. typhimurium* LB5010, followed by selection of the transformed strain on agar containing ampicillin.

The second problem was the maintenance of the LPS phenotype of *S. typhimurium* BRD509 during transformation. Straightforward heat-shock transformation can occasionally result in the loss of the O-antigen region of the LPS in the recipient bacterial strain. This gives the bacteria a 'rough' phenotype and allows antibodies and complement to bind to the bacteria enabling rapid clearance and reduced exposure of the antigen, rendering the vaccine less effective (Ernst *et al.* 2001; Freudenberg *et al.* 2001). Therefore an alternative method of transformation

was employed; the *Salmonella* specific bacteriophage P22 was used as a transfer vector between the two strains (as previously described by (Miller *et al.* 1989). Transformed *S. typhimurium* LB 5010 were infected with the phage. Due to the random nature of the packaging of DNA into virus particles, some of the resultant phage particles contain the plasmid instead of the P22 genome. The packaged DNA was then used to infect BRD 509 and any infectious particles were prevented from replication by addition of EGTA to the medium. Those bacteria in which plasmid had been successfully injected were then identified by their ability to grow on plates containing ampicillin. The presence of the plasmid within these clones was confirmed by PCR.

3.4.2.8 Characterisation of S. typhimurium BRD509 PGA-1

To verify that this transformed strain (designated BRD509 PGA-1) was able to express HBcAg, the recombinant bacteria were grown under anaerobic conditions and antigen expression confirmed by Western blot (Figure 3-7). The band identified by HBcAg specific sera was approximately 28kDa in size. This is larger than previously observed HBcAg from *P. pastoris* (see Figure 3-1) and reflects the addition of a number of amino acids upstream of the start codon of the protein. These codons do not affect particle formation but have been shown to enhance the level of expression of the recombinant protein in *E. coli* (D. Rowlands, personal communication).

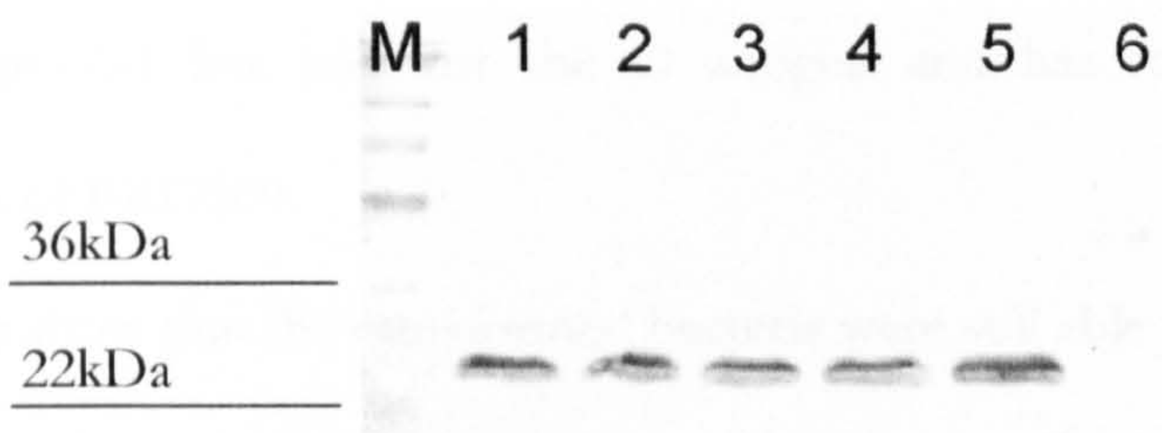


Figure 3-7: Expression of HBcAg from *S. typhimurium* BRD509 pGA-1

Anaerobic overnight cultures of *S. typhimurium* BRD509 pGA-1 were grown and a volume of culture that would give an OD_{650} reading of 0.5 was centrifuged and the pellet resuspended in protein sample buffer. These samples were run on a 30% acrylamide gel, transferred to nitrocellulose and visualised using polyclonal mouse anti-HBcAg antibody and HRP-conjugated rabbit anti-mouse antibody. Samples 1 – 5 are *S. typhimurium* BRD509 pGA-1. Sample 6 is BRD509 without the plasmid.

To ensure that the LPS profile of the transformed bacteria was intact, the bacteria were treated with proteinase K to remove the protein component and the remaining material was subject to SDS PAGE. The resultant gel was silver stained and the results shown in Figure 3-8. This figure shows the banding pattern associated with smooth LPS structure of *S. typhimurium* with typical core and O antigen sugars visualised. Comparison of the marker lanes (LPS samples lacking O antigen) with lane 1 (the original vaccine strain BRD509) illustrates the typical ladder feature associated with O antigen expression. From this figure, it would appear that no differences exist between the LPS profiles of BRD509 and LPS from the transformed strain BRD509 pGA-1 (lanes 2 and 3). Therefore *S. typhimurium* BRD509 pGA-1 has not lost the O antigen and has maintained the smooth phenotype of BRD509.

To show that the transformed bacteria were still able to invade and persist in eukaryotic cells, an invasion assay in a macrophage cell line, J774, was conducted using a similar protocol to that described previously (Jones *et al.* 1993). Over a period of 72 hours, the number of viable bacteria recovered from infected J774 macrophages was recorded. These data are shown in Figure 3-9 and indicates that the presence of the pGA-1 plasmid within the *Salmonella* strain does not affect the ability to both invade and persist in this cell line. Invasion of around 1% of the original inoculum occurs with both strains within the first 2 hours (and is in line with similar studies using this strain in the literature (Carlson *et al.* 2000)) and this level was maintained during the remaining 72 hours.

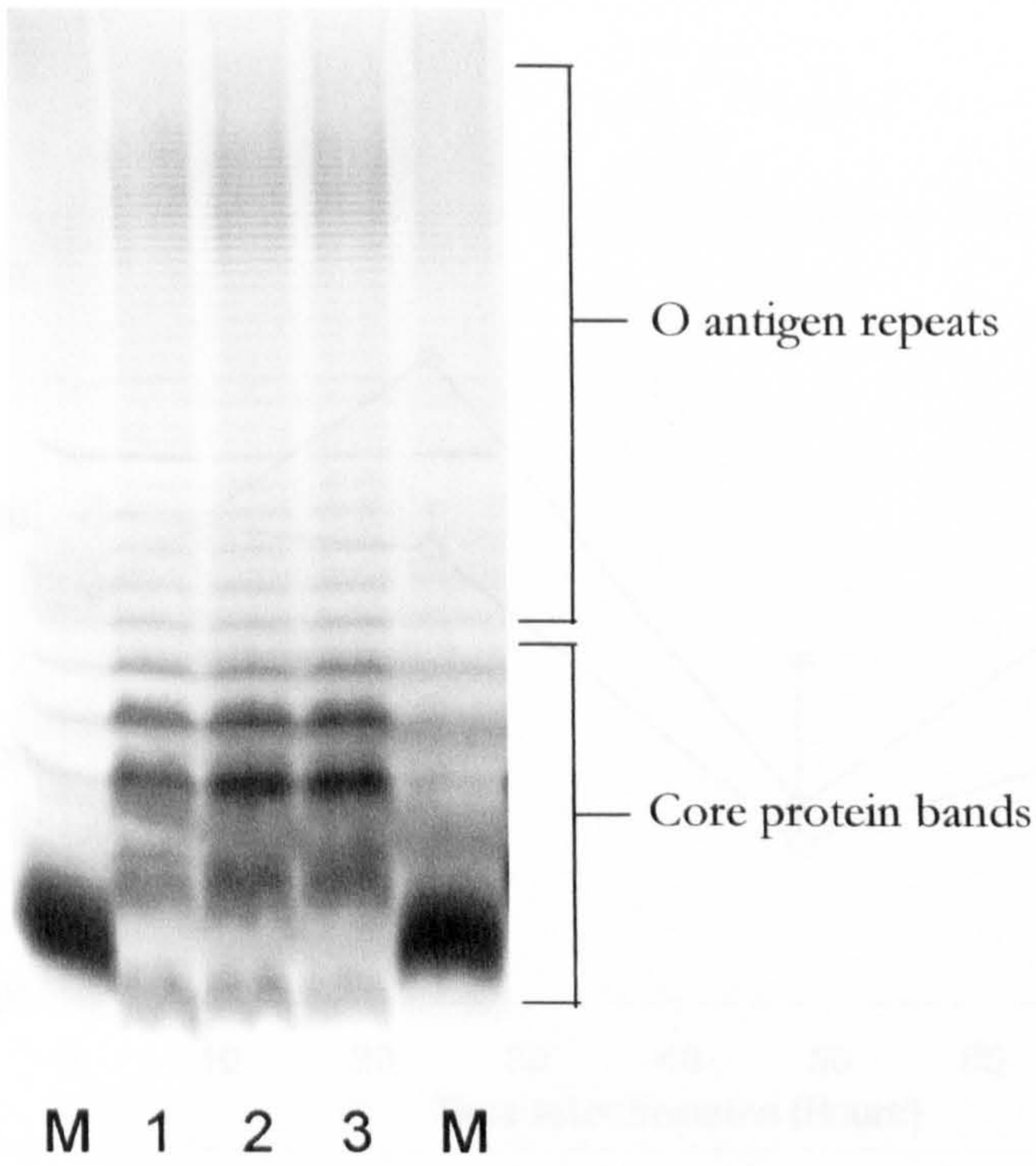


Figure 3-8: Silver-stained polyacrylamide gel comparing LPS from samples of *S. typhimurium* BRD509 and *S. typhimurium* BRD509 pGA-1

This gel confirmed that the LPS profile is unchanged in *S. typhimurium* BRD509 following transformation with bacteriophage P22 containing the plasmid pGA-1. Lane 1 contains LPS from BRD509, lanes 2 and 3 contain BRD509 pGA-1 LPS. M are markers containing LPS without O antigen.

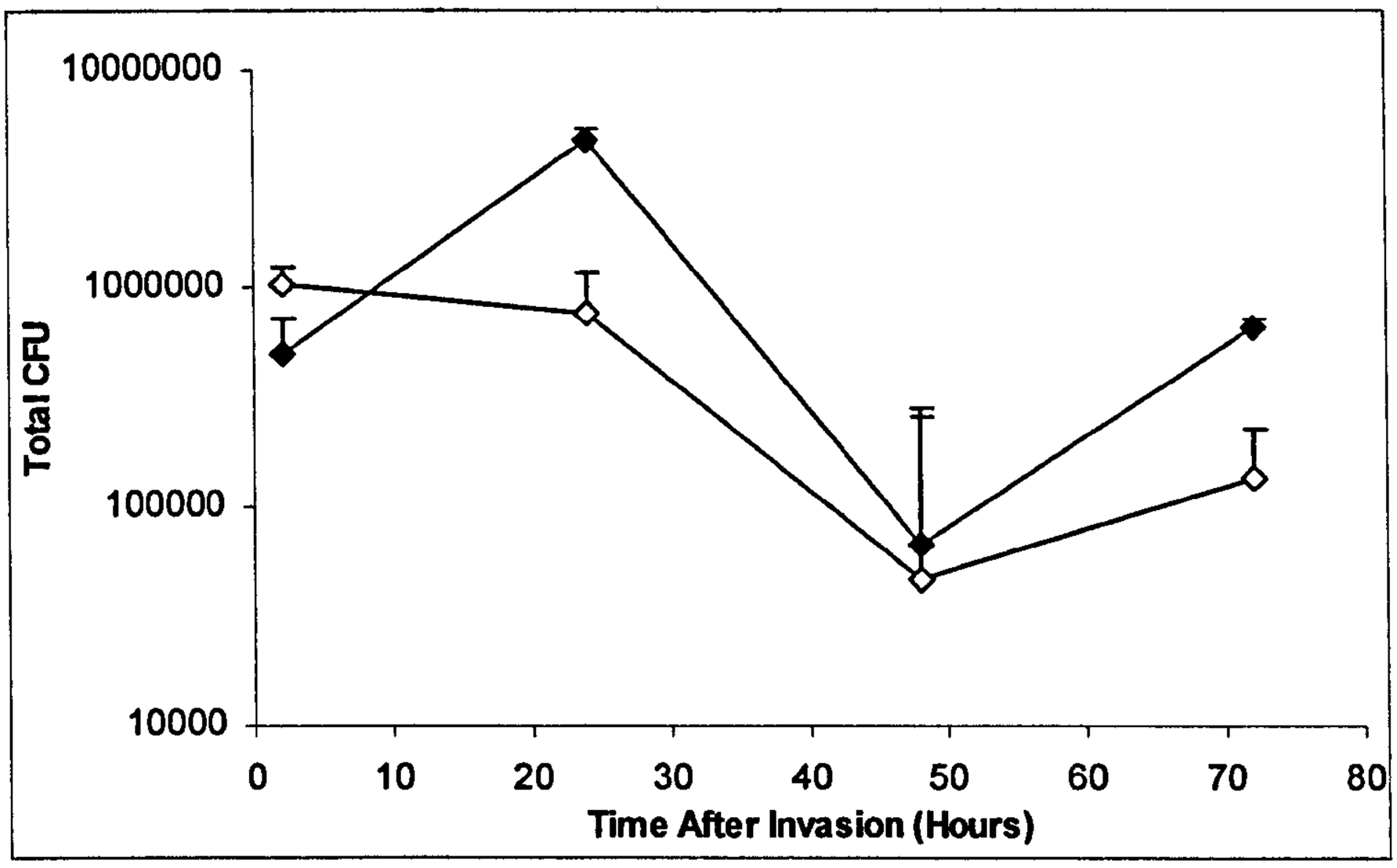


Figure 3-9: Number of bacteria recovered from infected J774 cells over time.

Following infection of J774 cells with the *S. typhimurium* BRD 509 strains, non-infecting bacteria were killed with the addition of gentamycin. At 2, 24, 48 and 72 hours after infection, cells were taken and lysed, the lysates plated out after serial dilution to give the number of infecting bacteria. ◇ represents BRD509 and ◆ represents BRD509 pGA-1.

Characterisation of *S. typhimurium* BRD509 pGA-1 clearly demonstrates that the bacteria are able to express HBcAg and possesses a smooth LPS phenotype. The vaccine is therefore judged as being suitable for *in vivo* vaccination experiments.

3.5 Summary

The work described in this chapter describes the characterisation of each vaccine type *in vitro*. It shows that each vaccine behaved as expected and could subsequently be used with some confidence for *in vivo* experiments.

4 RESULTS – ASSAY DEVELOPMENT

4.1 Chapter Aims

The aim of the work described in this chapter was to develop immunological assays capable of measuring both humoral and cellular immune responses to HBcAg following vaccination of mice. Optimisation of the procedures used ensured the reproducibility and sensitivity of the assays.

4.2 Introduction

As the overall objective of this thesis was to determine the influence of route and form of immunisation on the magnitude and quality of the immune response induced, it was necessary from the outset to define which aspects of the immune response should be measured. As the emphasis was on vaccine design, it was felt that such measurements be limited to aspects of the acquired immune response, in particular, to aspects of both the cellular and humoral response. The work outlined in this chapter describes the assays chosen for measurement of these responses and the modifications made in order to maximise the sensitivity and reproducibility of each method.

4.3 Humoral Immune Responses

Antibodies generated by B cells have a vital role in combating infection and protecting the host, through a number of functions including neutralisation and opsonisation of pathogens, activating complement and inducing NK-mediated

killing of infected cells. Antibody responses are important in the control of pathogens and toxins in the extracellular spaces of the body, including the mucosal surfaces, but through NK-mediated killing can also attack intracellular pathogens (see Introduction section 1.4.3).

One of the oldest and simplest forms of measuring an immune response is to assay the quantity of serum antibody against a particular antigen. The assay used to measure serum antibody levels was an ELISA.

4.3.1 Development of an HBcAg-Specific ELISA

The sensitivity and reproducibility of an antigen specific ELISA can be affected by a number of factors, which were considered when optimising this assay.

These include:

a) The initial concentration of coating antigen added to the wells of the assay plate. Insufficient antigen bound to the surface of the well will reduce the upper detection limit of the assay, even when the quantity of antibody in the sample is still increasing.

b) The coating buffer, in which the coating antigen is suspended, can affect the quantity of antigen bound to the plate; some antigens fail to bind effectively to plastic at neutral pH's, binding more effectively in alkaline conditions.

c) The selection of blocking agent. This prevents the non-specific binding of unrelated antigens to the surface of the well. An inappropriate choice of blocking buffer can allow other antigens to bind, potentially increasing the background levels of substrate colour change and decreasing the overall sensitivity of the assay.

d) Concentration of horseradish peroxidase (HRP) conjugated secondary antibody. HRP is the enzyme that catalyses the change of colour when the reaction substrate (O-phenyl diamine – OPD) is added. Since the colour change is proportional to the quantity of HRP present, it is essential to use sufficient to allow full detection of antigen-specific antibody bound to the wells.

These variables were examined to determine the optimal conditions that would allow clear and consistent assessment of antibody concentrations in different samples. In these optimisation experiments, a polyclonal mouse anti-HBcAg antibody raised by i.m. vaccination of mice with HBcAg in alum was used. This was known to contain a high concentration of antigen specific antibody and was therefore easy to detect.

For all experiments, the ELISA was performed as described in Materials and Methods section 3.5.1.1, with variations outlined below.

4.3.1.1 Coating Antigen Concentration

Wells were coated with a range from 0 to 1µg/ml of the coating antigen, HBcAg. As the concentration of the coating antigen bound to the well is also dependent on the buffer in which the antigen is added, the antigen was coated independently into different plates, the first using carbonate buffer as the diluent and the second using PBS. The plates were blocked in 10% NCS and polyclonal mouse anti-HBcAg was added at 2µl/well. After incubation, a secondary antibody (rabbit anti-mouse HRP conjugated antibody) was added at a 1:1000 dilution. Following addition of the substrate, colour was allowed to develop before the addition of acid to stop the enzyme activity. The colour change in the wells was then measured at a wavelength of 490nm and the absorbance recorded.

From this experiment, it appears that 0.5µg HBcAg saturated the wells of the plate. Figure 4-1 shows the effect of coating the wells of the ELISA plate with different concentrations of HBcAg. Figure 4-1A shows the absorbance from wells coated with antigen in carbonate buffer and 4-1B shows that of wells coated using PBS. The choice of buffer had a small impact on the assay sensitivity, with those wells coated with HBcAg in carbonate buffer showing a higher OD

4.3.1.2 Selection of Blocking Buffer

For blocking of non-specific binding to the wells, two buffers were considered; Newborn Calf Serum (NCS) and BSA. Wells were coated with 0. 5µg/ml HBcAg in carbonate buffer. Two plates were then blocked; one with 10% NCS and one with 1% BSA, for 1 hour at 37°C. The results are shown in Figure 4-2A (NCS) and 4-2B (BSA). These indicate that the buffers function similarly well. Since the NCS was relatively inexpensive compared to BSA, 10% NCS was used as the blocking agent in all subsequent work.

4.3.1.3 Concentration of Anti-Mouse HRP Conjugated Antibody

To determine the effect of varying the concentration of the conjugated antibody, various concentrations ranging from 1:250 to 1:5000 were used to detect the mouse antibody bound to HBcAg on the plates. Over the range of dilutions examined, a dilution of 1:1000 was shown to provide greatest sensitivity.

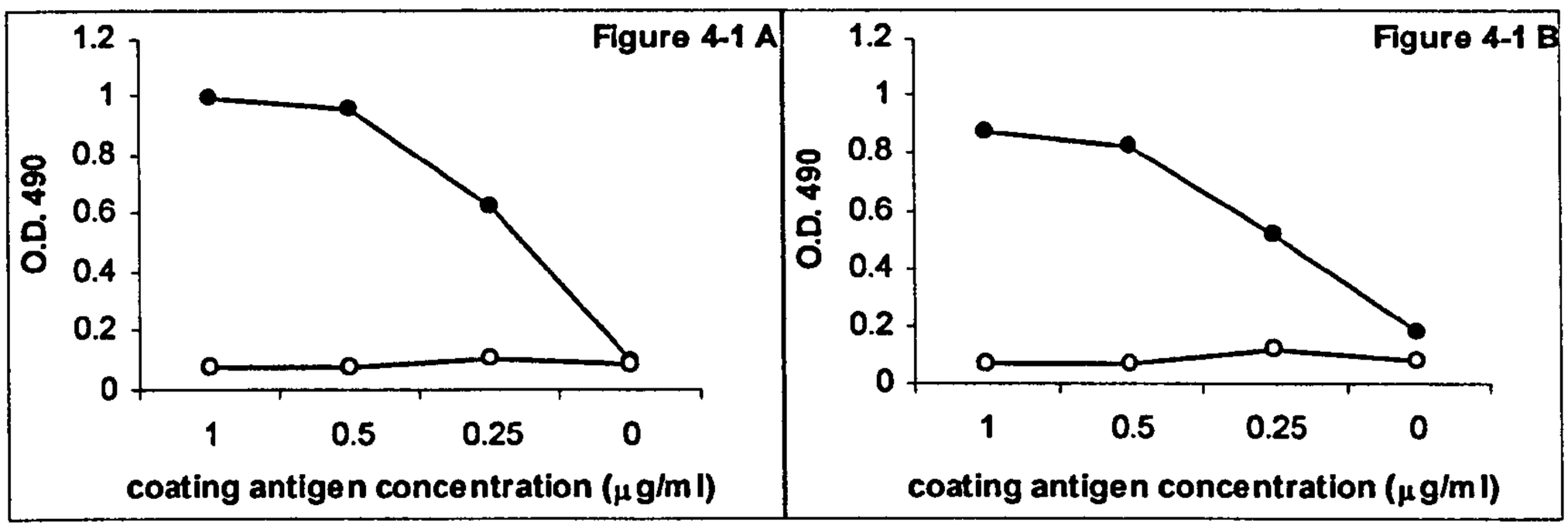


Figure 4-1: Impact of coating antigen concentration and buffer on HBcAg-specific ELISA

Wells were coated with different concentrations of coating antigen (HBcAg) in carbonate buffer (Figure 4-1A) or PBS (Figure 4-1B) and blocked with NCS. HBcAg was bound with polyclonal anti-HBcAg sera and detected with a 1:1000 dilution of HRP conjugated secondary antibody. ● represent ODs from reactions containing secondary antibody at a 1:1000 dilution. o represent ODs from reactions without secondary antibody.

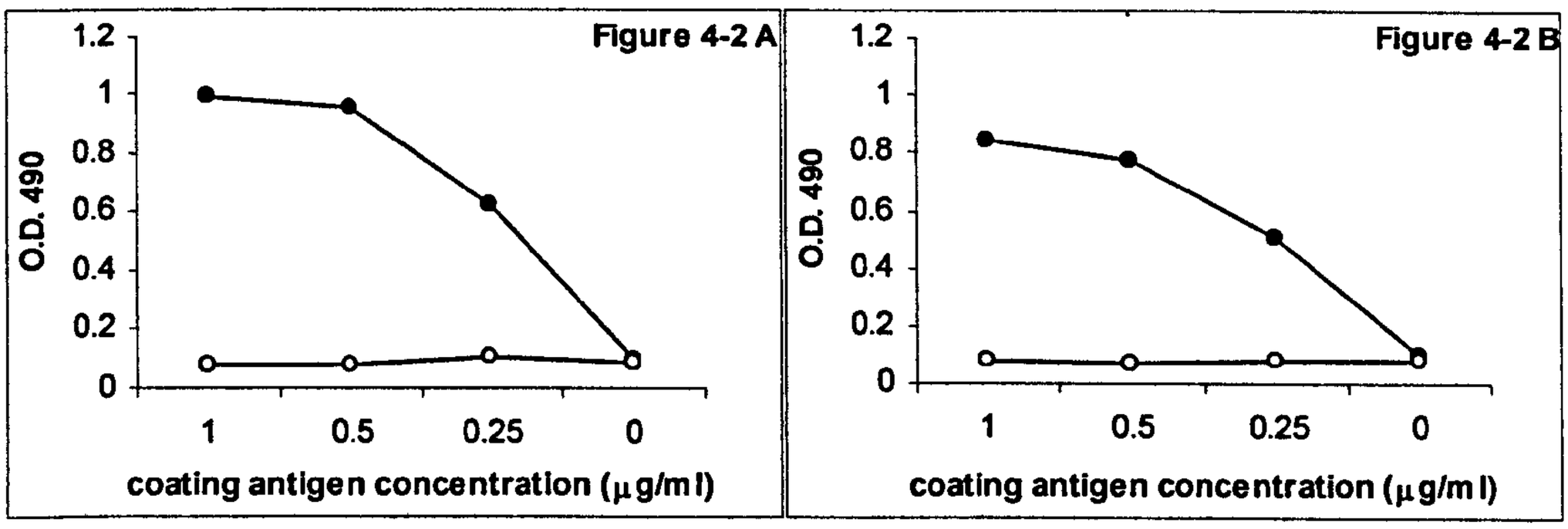


Figure 4-2: Impact of blocking agent on HBcAg-specific ELISA

Wells were coated with different concentrations of HBcAg in carbonate buffer, blocked with 10% NCS (Figure 4-2A) or 1% BSA (Figure 4-2B). HBcAg was bound with polyclonal anti-HBcAg sera and detected with a 1:1000 dilution of HRP-conjugated secondary antibody. Negative control values (no anti-HBcAg sera added) were subtracted from the values shown. ● represent ODs from reactions containing secondary antibody at a 1:1000 dilution. o represent ODs from reactions without secondary antibody.

4.3.1.4 Optimised HBcAg-Specific ELISA

To summarise, the final ELISA assay consisted of plates coated with 50µl of 0.5µg/ml HBcAg in sodium carbonate buffer. Plates were blocked with 10% NCS. After the test antibody samples had been added, secondary antibody was added to wells at a dilution of 1:1000. The plates were developed with OPD substrate and the OD 490 was measured. Between each step plates were incubated for 2 hours at 37°C and washed thoroughly in PBST. The full protocol is detailed in Materials and Methods section 2.5.1. Data were analysed using Microsoft Excel and variations between separate ELISA plates were corrected using the titres of a standard positive control.

4.3.2 ELISA Subtype Assay

The method for measuring titres of individual IgG subtypes was similar to that of the total immunoglobulin ELISA. The main difference was the use of subtype-specific secondary antibodies: anti-IgG1 and anti-IgG2a. These antibodies were biotinylated, so the plates were additionally incubated with 1:1000 dilution of streptavadin-HRP for an hour at 37°C prior to the final wash. The anti-isotype antibodies were added to wells at dilutions of 1:5000 for IgG1, and 1:2500 for IgG2a, as these antibodies had been shown in previous studies performed in this laboratory to have a slightly different avidity for each isotype. The dilutions used have been calculated as capable of detecting the equivalent amount of each of the respective IgG isotypes in a mixed population.

4.3.3 Measurement of HBcAg-Specific IgA

Immunoglobulin A (IgA) is a class of antibody important in protecting against infection and pathology at mucosal surfaces, as described in the Introduction, section 1.4.3. In order to develop an ELISA that could be used to determine the IgA levels in different samples, it was necessary to a) employ a method that would discriminate between IgA and other immunoglobulins and b) obtain a positive control IgA sample that would allow comparison between ELISA plates. In order to generate a positive control for IgA-specific ELISAs, mice were immunised i.n. with 2µg HBcAg and 2µg *E. coli* labile toxin (LT) in a volume of 30µl PBS. They received an identical boost 10 days later. After a further 10 days, the mice were sacrificed and washes of the lungs and nasal cavity were made, using sterile PBS containing 1% BSA. These mucosal washings were pooled and used as positive control for these assays. The IgA ELISA was conducted in the same way as that described for total antibody (section 4.3.1.4), except for the use of IgA specific secondary antibody. The assay is described in full in Materials and Methods sections 2.5.1.2).

4.4 T Cell Responses

T lymphocytes (T cells) fall into two main functional classes that can be distinguished by their cell-surface markers; cytotoxic T lymphocytes (CTLs) bear a cell surface molecule called $CD8^+$ and helper T (Th) cells carry the $CD4^+$ molecule. The activation of these cells is an important indicator of the immune response.

4.4.1 *Ex Vivo* Measurement of HBcAg-Specific $CD4^+$ T Cell Proliferation

$CD4^+$ Th cells enhance antibody function, phagocytosis by macrophages and CTL activation (see Introduction section 1.4.2.2). The presence of a Th cell population able to proliferate in response to an antigen is an indicator of effective vaccination, and is therefore an important aspect of the response to be measured.

Following successful vaccination, antigen-specific $CD4^+$ T cells differentiate and proliferate. The higher the number of these cells present in the animal tissue, the greater the level of *ex vivo* proliferation seen upon restimulation of cells from the lymphoid tissue with antigen. The proliferation of cells can be measured by the addition of radioactively labelled nucleotides to the media. These molecules are taken up by cells and used in the synthesis of DNA when they divide, therefore the level of radioactive uptake is proportional to the level of proliferation in the culture. The level of uptake is measured by harvesting the cells, washing off free radioactive nucleotides still in the media and assaying the cells for β-particle emission. This forms the basis of the proliferation assay. The sensitivity of the assay depends on a variety of factors outlined below.

a) Culture media selection, specifically foetal calf serum (FCS). FCS is an essential component of tissue culture media used to maintain the immune cells during the course of the proliferation assay. Different batches of FCS can contain different levels of mitogenic components. These compounds can cause the non-specific proliferation of cells in the culture. Since any cell synthesising DNA will take up radioactive nucleotides, regardless of the initial stimulus for proliferation, the assay does not differentiate between specific and non-specific proliferation. Therefore to ensure the assay

measures antigen-specific proliferation only, it is essential to select a batch of FCS with minimal mitogenicity.

b) Selection of restimulating antigen. A complication of using HBcAg as the model antigen in these experiments, is the fact that it can act as a T cell independent B cell mitogen (Milich and McLachlan 1986). This can be another cause of non-specific proliferation of B-cells in the assay culture, making measurement of $CD4^+$ antigen-specific proliferation impossible. One way around this problem is to use a suitable $CD4^+$ peptide on the HBcAg as the restimulating antigen. This will be appropriate for the restimulation of existing HBcAg specific $CD4^+$ cells without causing non-specific proliferation of B-cells.

c) Cell population selection. Another means of avoiding non-specific proliferation of B cells is to remove these cells from the proliferation culture. This can be done using MACS separation (see Materials and Methods section 2.5.3) in one of two ways: by selectively removing the B cells from the population, or by selecting only the $CD4^+$ cells for inclusion in the proliferation assay. The second choice requires the addition of an APC population to the culture.

The aim of the following experiments was to determine the impact of the factors described above on the execution of an antigen-specific T cell proliferation assay. The results were used to develop an optimised assay that would be able to reproducibly demonstrate quantitative differences in the responses generated by the different vaccine regimes examined later in this thesis.

4.4.1.1 Generation of Immune Cells for Development of the HBcAg-Specific CD4⁺ T Cell Proliferation Assay

To generate populations of HBcAg-specific T cells, mice were immunised i.m. with 5μg of HBcAg in alum or s.c. with 3μg of HBcAg in CFA (actual doses and adjuvants are described in each figure legend). Ten days after vaccination, the spleens of immunised animals were removed and single cell suspensions prepared (as described in Materials and Methods section 2.5.2). The cells were then used either directly in the proliferation assay or separated by MACS (Materials and Methods section 2.5.3) prior to inclusion in the assay cultures. The mouse strain used in these experiments was C57/Bl6.

4.4.1.2 Media Selection

To identify an appropriate source of FCS for use throughout the entire project, two batches of FCS, obtained from different companies (Gibco LifeSciences and Harlan Serolabs), were compared. Media was prepared containing 10% FCS and used to set up proliferation assays using cells taken from immunised and naïve mice. For both groups of animals four spleens were initially pooled and single cell suspensions prepared. As purified HBcAg was to be used as a restimulating antigen in this assay, B-cells were removed from the population using the MACS system. The resultant cell suspension was divided into two equal volumes of cells and the assay was performed in parallel using media containing the different batches of FCS. Apart from the media used, the assay was performed as described (Materials and Methods 2.5.4).

The impact of using different batches of FCS is shown in Figure 4-3. This figure shows the proliferation of B cell depleted spleen cells from mice immunised with HBcAg and CFA compared to non-immunised controls. For these

experiments cells were resuspended in media containing Gibco FCS (Figure 4-3A) or media containing Harlan FCS (Figure 4-3B). This shows that proliferation occurs at similar levels whichever source of FCS is used and suggests that use of one particular sera does not provides a significant advantage. However, when the proliferation from non-immunised animals is considered, the nonspecific response is seen to be lower and less varied when the Harlan FCS was used.

Therefore, to minimise the level of non antigen-specific proliferation in the assay, and to decrease variation within groups, a large batch of Harlan FCS was purchased and used in all subsequent experiments described in this thesis.

4.4.1.3 Peptide Versus Protein as Restimulating Antigen

To determine whether HBcAg or a small $CD4^+$ specific peptide from HBcAg acts as a more effective restimulation antigen, the two antigens were compared in parallel. For this assay, the splenocytes from immunised and non-immunised animals were isolated and the B cells removed by MACS (Materials and Methods 2.5.2). The cells restimulated with titrating concentrations of either the whole protein or a previously described HBcAg $CD4^+$ peptide for H2-b mice (Milich *et al.* 1987). The sequence for this peptide is given in Materials and Methods Table 2-3. The cells were restimulated with concentrations of peptide that had previously been shown to be effective in this paper. The results of this experiment are shown in Figure 4-4.

The three major points of interest about these data are:

1) Both the HBcAg and $CD4^+$ peptide are able to stimulate antigen specific T-cells in the spleen cell population to proliferate in a concentration dependent manner.

2) The thymidine incorporation is approximately 3 fold higher in the peptide-stimulated cultures than in the equivalent HBcAg cultures, in both immunised and naïve groups.

3) The immunised cells in the peptide cultures respond much more strongly to an increase in antigen concentration than the cells in the equivalent HBcAg culture, over the range of concentrations examined.

From initial consideration of the data, it is not obvious whether protein or peptide restimulation better provides a sensitive and consistent proliferation assay. In terms of counts measured, the ratio of immunised to naïve counts was the same for both peptide and protein. The only important difference between the two was that small increases in the peptide concentration had a greater effect on the level of proliferation than the equivalent changes in HBcAg concentration. However, in terms of practical application, use of the peptide has another advantage in that it does not require the removal of B-cells.

For these reasons, it was decided that the $CD4^+$ peptide is a more effective restimulation antigen than the entire HBcAg, and therefore restimulation using this peptide was adopted in all future experiments.

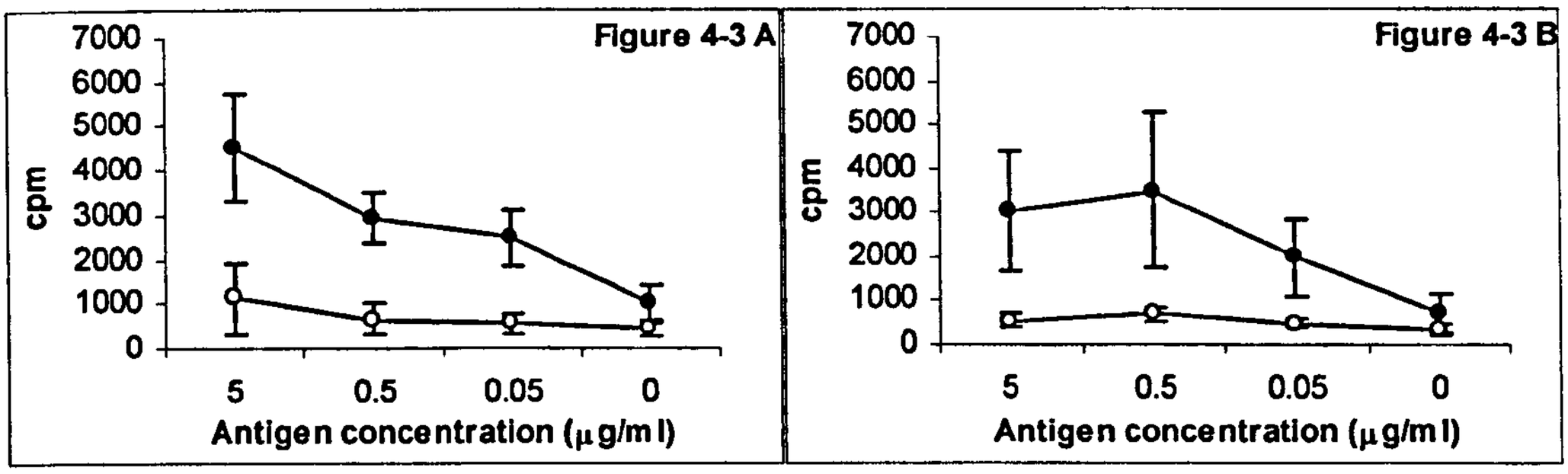


Figure 4-3: Impact of different sources of FCS on the proliferation of HBcAg-specific T cell responses.

C57/Bl6 mice were immunised s.c. with 3µg HBcAg in CFA. The proliferation of a pool of B cell depleted spleen cells from 4 mice was measured by β-emission of cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of HBcAg concentrations. Mean CPM of triplicate cultures of pooled cells are shown ± 1SD. The two cultures were restimulated with media containing HBcAg with FCS sourced from Gibco life sciences (Figure 4-3A) or from Harlan serolabs (Figure 4-3B). CPM from cells of immunised mice are represented by ●. CPM from the cells of naïve mice are represented by o.

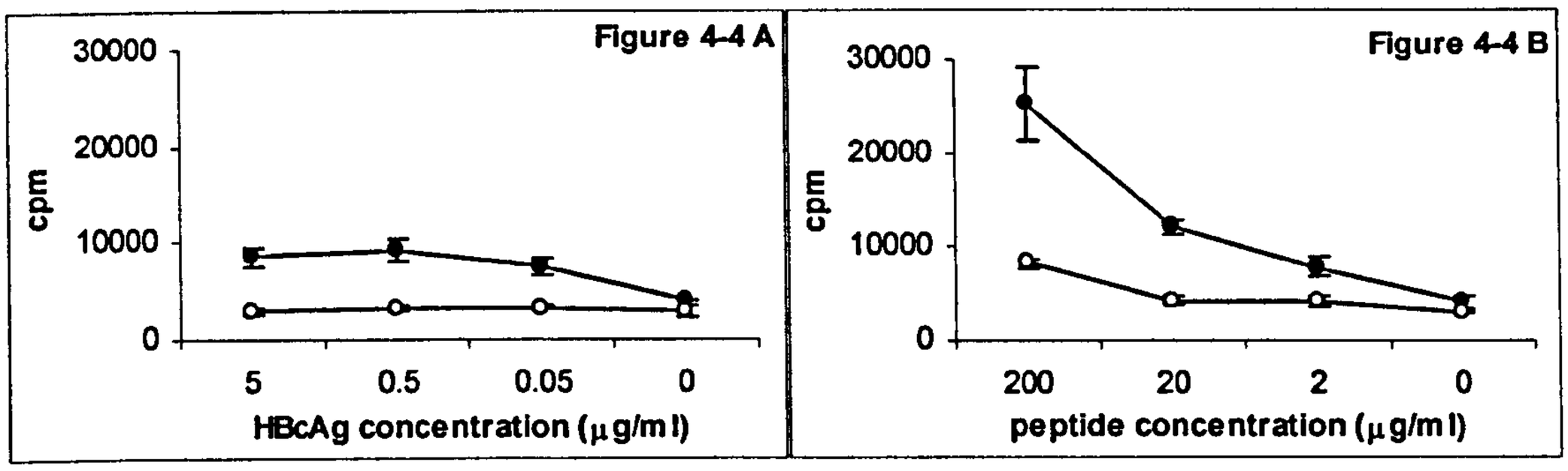


Figure 4-4: Impact of using purified protein versus HBcAg p120-140 on proliferation of HBcAg specific T cells

9 C57/Bl6 mice were immunised i.m. with 5µg HBcAg in alum on day 1 and day 8. Mice were sacrificed and splenocytes recovered on day 15. Proliferation of spleen cells from pooled mice was measured by β-emission from cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of antigen concentrations. Mean CPM from triplicate cultures of pooled cells are shown ± 1SD. Figure 4-4A shows cells cultured with HBcAg as the restimulating antigen, whereas Figure 4-5B shows cells restimulated with peptide HBcAg p120-140. CPM from cells of immunised mice are represented by ●. CPM from cells of naïve mice are represented by o.

4.4.1.4 Cell Population Selection

As the T cell proliferation assay measures proliferation of all cells in the culture, irrespective of the stimulus or cell type, it is vital to the accuracy of the assay to prevent both non-specific T cell proliferation, and proliferation of other cell types.

One approach employed to avoid the problem of B cell mitogenicity involves the removal of these cells from the rest of the lymphocyte population. This can be accomplished using the MACS separation system (Materials and Methods section 2.5.3). The choice of antibody used in the system determines which cells are removed and which remain in the population. Two different methods were considered.

In the first method, the B cells alone were removed using a magnetic anti-CD19 antibody. As CD19 is a B cell marker, use of this antibody causes B cells to become stuck to the magnetic column whilst the remaining splenocyte population is able to pass through uninhibited. The advantage of this method is that it does not remove the majority of APCs present in the spleen that are required for efficient presentation of the antigen *ex vivo*.

In contrast, a second method makes use of a $CD4^+$ specific antibody attached to a magnetic bead, which binds the $CD4^+$ T-cells to the column. The resulting, highly pure, population of $CD4^+$ T cells can then be eluted by removal of the magnet and should not be contaminated with B cells. However, to be effective in the proliferation assay, the HBcAg specific T cells require presentation of the antigen by a suitable APC. For these experiments, 3 different types of APC were considered. These were:

1. Irradiated splenocytes. Spleen cells were taken from a naïve mouse and irradiated. These cells are still able to take up and present antigen but

are unable to proliferate due to the damage caused to their DNA by irradiation. Cells were used at a ratio of 1:3 irradiated splenocytes : T cells in the proliferation assay.

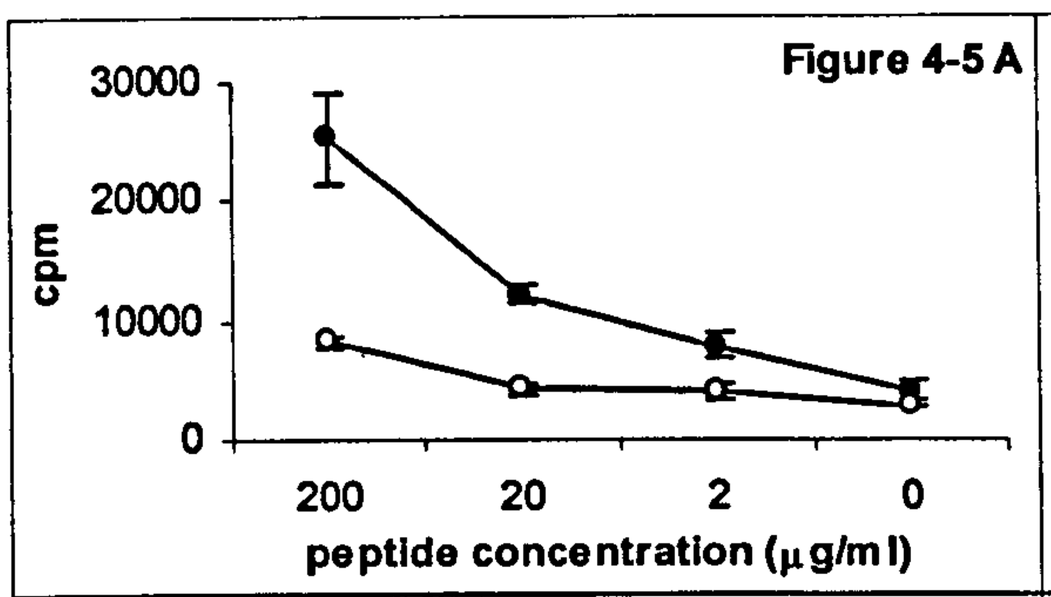
2. Mitomycin C treated splenocytes. Mitomycin C treatment causes DNA alterations to the cell that again prevents proliferation without inhibiting antigen presentation. Cells were treated with 50μg mitomycin C per 10^7 splenocytes for 75 minutes and washed thoroughly to remove the mitomycin C before being added to cultures at a ratio of 1: APCs : T cells (see Materials and Methods section 2.5.4.2).
3. Irradiated naïve DCs. DCs are potent specialist antigen presenters, and can be cultured from the bone marrow cells of mice stimulated with granulocyte-monocyte colony stimulating factor (GM-CSF) (Materials and Methods section 2.5.4.3). The process of irradiation was the same as that used for splenocytes. Cells were added at 1:3 APCs : T cells.

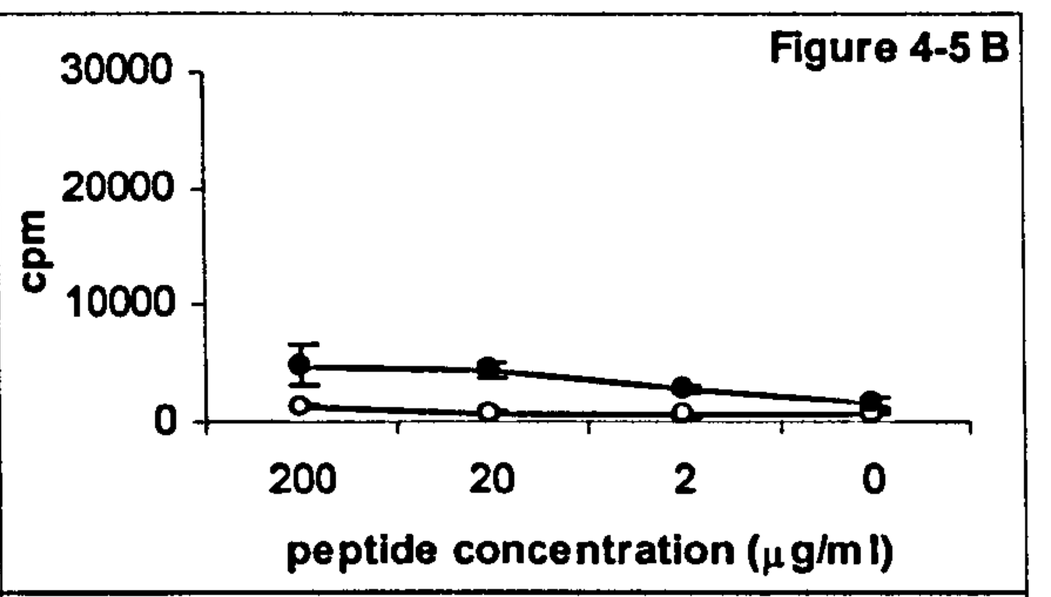
To find the optimal cell population to use in the proliferation assay, C57bl/6 mice were immunised with HBcAg in alum, as described above in section 4.4.1.2. Cells were recovered from the spleens of vaccinated animals and split into four populations of equal number. One volume of cells was depleted of B cells using anti-CD19 beads. The remaining populations were treated with anti-$CD4^+$ beads and the $CD4^+$ cell population isolated. These were then used to set up three proliferation assays, each provided with one of the APC types described above. All four cultures were then restimulated with the $CD4^+$ peptide as the restimulating antigen.

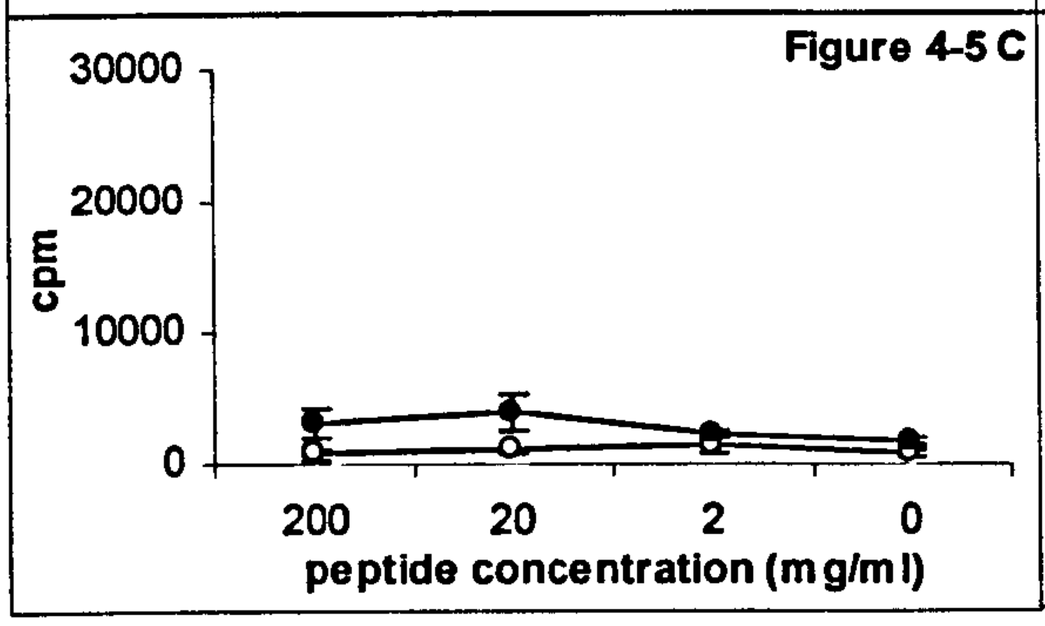
The results of these experiments are shown in Figure 4-5. In all cases, HBcAg specific $CD4^+$ proliferation was induced with higher counts in wells

containing cells from immunised animals than those from naïve animals. The proliferation observed was also shown to be antigen-concentration dependent. However, the greatest proliferation was observed when only the B cells were depleted from the cell suspension; Figure 4-5A. This is despite the higher level of emission from naïve groups in this experiment compared to those using non-proliferating splenocytes; both the absolute and proportionate increase of the immunised group over the naives is greater than in any other group. Also, the immunised B cell depleted cultures were more sensitive to increases in antigen concentration than any other group.

One factor that might explain the high level of proliferation in this system may be that the APCs in the B cell depleted culture (Figure 4-5A) have not suffered significant DNA damage. Irradiation and mitomycin C treatment both result in significant damage to chromosomal DNA and prevent the cells replicating. Protein expression from damaged genes will also be impaired, and although the cells are able to survive and present antigen, this may be less efficient than in non-treated cells (Figure 4-5B and C).







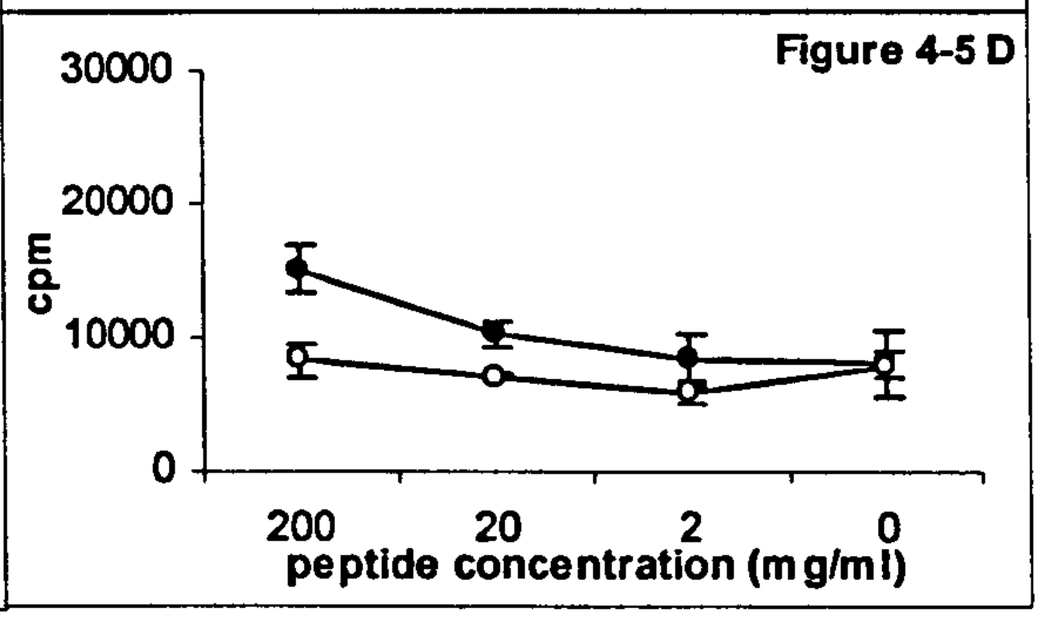


Figure 4-5: Comparison of proliferation of T cells with different APC populations.

9 C57/Bl6 mice were immunised i.m. with 5µg HBcAg in alum. The proliferation of a pool of spleen cells from 9 mice was measured by β-emission from cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of antigen concentrations. Mean CPM from triplicate cultures of pooled cells are shown ± 1SD. Figure 4-5A shows B cell depleted splenocytes, while B, C and D show $CD4^+$ T cells with added APCs. Figure 4-5B shows T cells plus naïve irradiated splenocytes, Figure 4-5C shows T cells with naïve, mitomycin C treated splenocytes and Figure 4-5D shows T cells with naïve, mitomycin treated DCs as APCs. CPM from cells of immunised mice are represented by ●. CPM from cells of naïve mice are represented by o.

After the B cell depleted group, the next highest level of proliferation was measured in wells in which purified $CD4^+$ T cells had been admixed with irradiated DCs (Figure 4-5D). DCs are potent APCs, capable of efficiently presenting antigen to T cells, which probably explains their success when compared to the mixed populations of naïve non-proliferating splenocytes present in the two remaining cultures.

A problem with the cultures containing DCs as APCs is the high level of proliferation seen in the naïve group. This indicates that either the DCs are causing T cells to proliferate non-specifically, or that the DCs were insufficiently treated with Mitomycin C to prevent their own replication during the proliferation assay. Whichever theory is correct, it means that these cells are not suitable for use in the proliferation assay.

As a result of these data, all further experiments looking at antigen specific $CD4^+$ T cell proliferation were performed using CD-19 B cell deleted lymphocyte populations.

4.4.1.5 Optimised Assay for Measuring HBcAg-Specific $CD4^+$ Responses

As a result of these experiments, $CD4^+$ T-cell responses were subsequently measured by isolation of a splenocyte population whose B-cells were removed using CD19 beads. To each well of a microtitre plate, $2x10^5$ cells were added and were restimulated with the HBcAg $CD4^+$ peptide (HBcAg p120-140). All RPMI media used in culturing of these cells contained FCS sourced from Harlan Serolabs. Cultures were incubated for 72 hours at 37°C, 5% CO_2, then pulsed with tritiated thymidine.

4.4.2 $CD8^+$ CTL Responses

CTLs are a second type of T lymphocyte, generally characterised by the presence of $CD8^+$ molecules on the cell surface, rather than $CD4^+$ as on T helper cells. Functionally, they are quite different from T helper cells. Although they also recognise antigen in a specific manner, antigen can be presented to $CD8^+$ T cells by almost any cell (not just specialist APCs), since they recognise antigen bound to MHC class I molecules, expressed by nearly every cell type in the body. On recognition of antigen and following a secondary signal, a naïve $CD8^+$ T cell is activated and differentiates into a CTL that can kill cells presenting its specific antigen by inducing apoptosis. This mechanism is used to control intracellular pathogens, such as viruses and invasive bacteria. Activated CTLs also secrete IFN-γ a molecule whose functions include the inhibition of virus replication within cells and the activation of macrophages (see Introduction section 1.4.2.1). Both CTL killing and IFN-γ secretion have been assayed for as part of this project.

4.4.2.1 CTL Killing Assay

In order to determine whether different formulations of vaccine could stimulate HBcAg-specific $CD8^+$ killing responses, a chromium release assay was developed. This CTL killing assay is based on the ability of antigen specific $CD8^+$ cells to recognise and destroy target cells which express the HBcAg specific $CD8^+$ peptide on their surface. The assay itself involves the isolation of splenocytes from vaccinated animals, which are incubated with the target cell population *in vitro* for 5 days. This is necessary to restimulate and amplify the number of specific CTLs generated. After 5 days, the amplified cell population is re-exposed to a fresh set of target cells that have been previously incubated with radioactive sodium chromate

[^{51}Cr]. After co-incubating radioactive cells with the CTLs for 4 hours, the culture supernatants are removed and measured for β-emission. As target cells that have been destroyed by CTL activity release [^{51}Cr] into the supernatant, the level of radioactivity in the supernatants can be correlated to the number of cells destroyed. This is calculated as a percentage by comparing the emission to populations of target cells lysed with a detergent, triton X, which gives 100% lysis, and unlysed target cells.

Optimisation of [^{51}Cr] Sodium Chromate Labelling of Target Cells

HBcAg specific target cells, P815/c and the equivalent P815 control cells were a kind gift from R. Schirmbeck, University of Ulm, Germany. To optimise the assay the uptake of radioactive sodium chromate [^{51}Cr] by these cells was examined. Different numbers of target cells were incubated for different lengths of time with radioactivity. To quantify the absorbance of radioactivity, cells were lysed with triton X 100 and the β emissions of the supernatants measured. The results are shown in Figure 4-6. The data shows that the β-emission increases with the number of cells labelled, but is not directly proportional, with a greater increase between $1x10^4$ and $1x10^5$ cells than between $1x10^3$ and $1x10^4$. The greatest increase in counts due to incubation time was between 30 minutes and 1 hour, with a longer incubation period giving less of an increase.

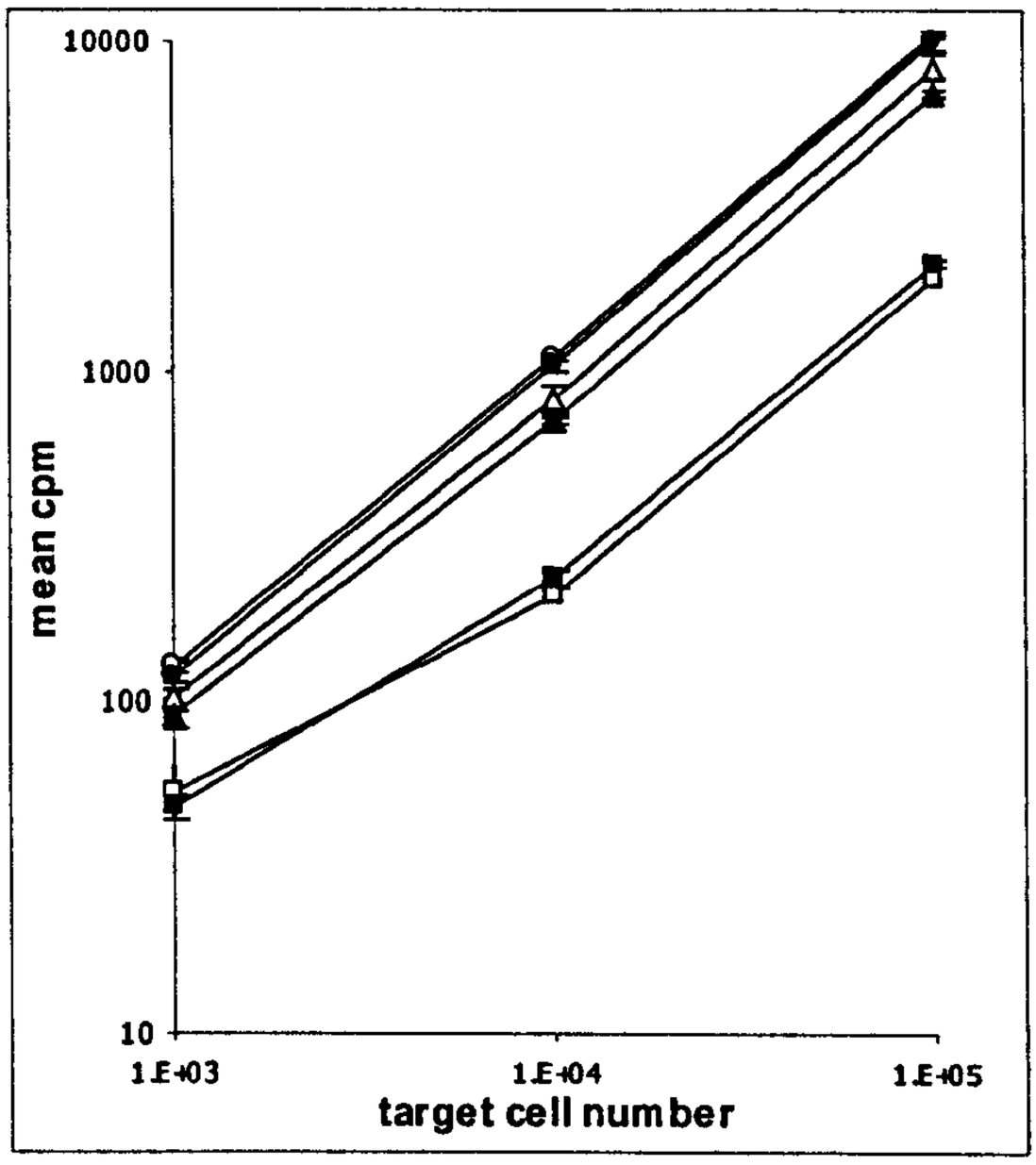


Figure 4-6: Effect of incubation time and target cell number on Cr^{51} absorbance

The amount of radioactivity absorbed by either P815 (hollow symbols) or P815/c cells (filled symbols) was measured by lysis of these cells with detergent after 30 minutes (■), 60 minutes (▲) or 120 minutes(●). Mean CPM of triplicate cultures are shown ± 1SD.

The results from this experiment show that $1x10^5$ target cells generate a strong β-emission signal, i.e. in the order of 10,000cpm. The counts were directly proportional to the number of cells present, and both target (P815c) and control (P815) cells gave similar counts. Therefore it was considered that $1x10^5$ target cells per well was the optimal number for use in these assays and cells should be labelled for a minimum of 1 hour prior to use.

Optimisation of the Chromium Release Killing Assay

To determine whether antigen specific $CD8^+$ responses could be measured *in vivo*, BALB/c mice were vaccinated with 100μg pcDNA3. 1/core i.m. and sacrificed after 10 days. Splenocytes were isolated and restimulated with target cells for 5 days, as described above. They were then incubated for 4 hours with sodium [^{51}Cr] chromate labelled target cells and the β emission measured.

Figure 4-7 shows the percentage of specific killing by cells from BALB/c mice immunised with pcDNA3. 1/core, pcDNA3. 1 and in untreated animals. These data show that the assay is able to measure antigen-specific killing, which occurs effectively in animals immunised with the pcDNA3.1/core vaccine. Such induction is in agreement with similar observations in the literature (Kuhrober *et al.* 1996). This assay appears discriminatory with little killing observed in control groups. It was therefore decided that this assay was sufficiently sensitive and discriminating to be used for measuring CTL activity.

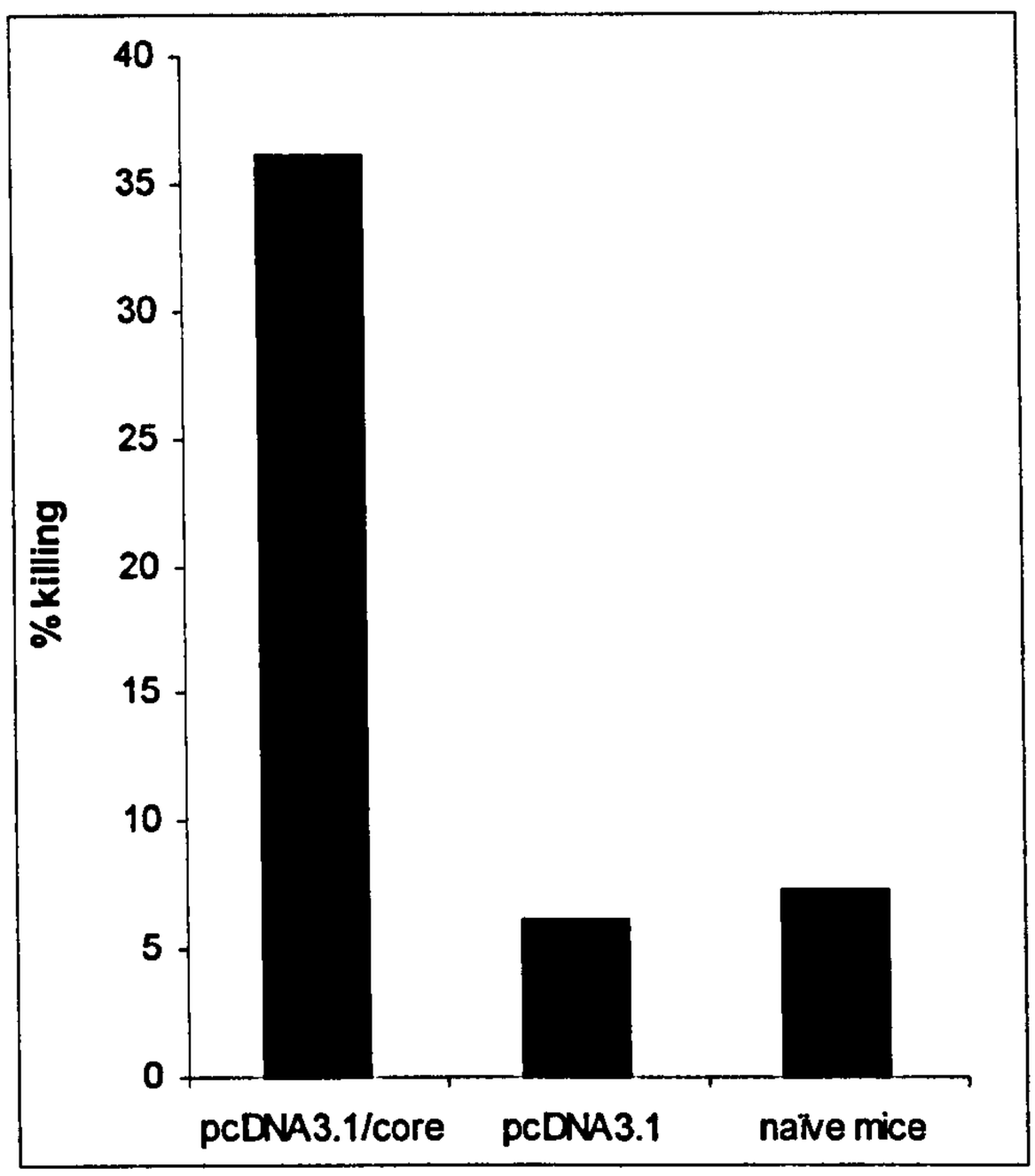


Figure 4-7: HBcAg-specific CTL killing by mice immunised with pcDNA3.1/core

Percentage of specific killing of target cells by spleen cells restimulated *in vitro* for 5 days, following harvesting from BALB/c mice 10 days after immunisation with pcDNA3.1/core. 50:1 effector : target cell ratio.

4.4.2.2 Interferon-γ Release – an Indirect Measurement of CTL Activation

One feature of CTL activation that can be used as an indirect measurement of CTL activity is secretion of IFN-γ. To assess whether CTLs could be quantified using this method, 5 BALB/c mice were vaccinated with 100μg pcDNA3. 1/core i.m. and sacrificed after 10 days. Using the protocol described in the materials and methods 2.5.7, adapted from (Culshaw *et al.* 1997), no spots were detectable on the filter, suggesting that no activated CTLs were present. However, considering that the CTL killing described in section 4.4.2.1 was so high, it was felt that the HBcAg specific CTLs were present, but that assay lacked discrimination. In order to emulate the method and level of response indicated by the killing assay, it was decided to restimulate the isolated T cells with target cells prior to their use in the ELISPOT (see above).

Therefore the isolated T cells were restimulated for 5 days in vitro at a ratio of 10 : 1 effector : target cells. Using this method clear, antigen specific IFN-γ production was observed. The numbers of Spot Forming Cells (SFC) are shown in Figure 4-8. The data in this figure confirms that immunisation of mice with pcDNA3.1/core results in the generation of IFN-γ producing T-cells. Although it is likely that the production of this cytokine is from antigen specific $CD8^+$ cells (given the use of the $CD8^+$ specific peptide), this could have been further confirmed by intracellular cytokine staining of these cells and measurement by FACSort analysis. This assay appeared to be discriminating as the number of spots generated following vaccination with pcDNA3.1/core was approximately twice the number observed in equivalent control wells.

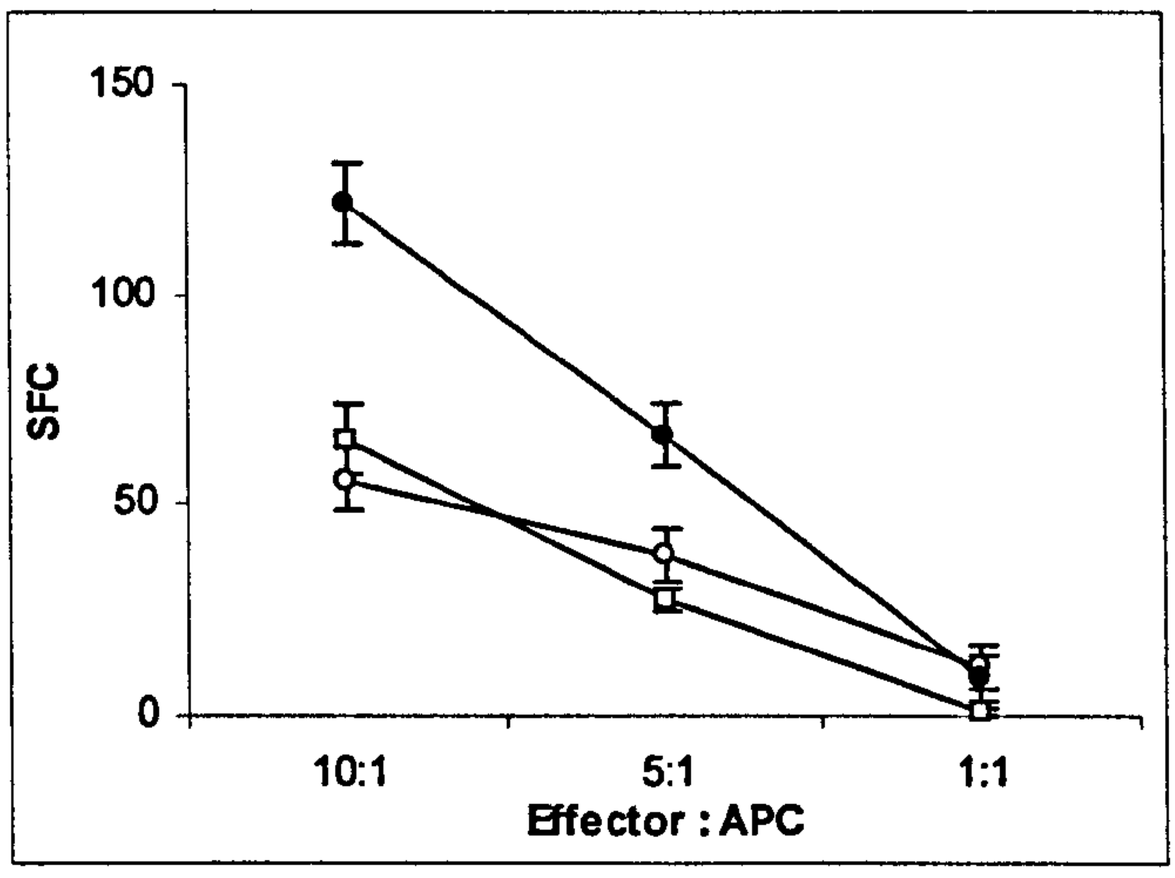


Figure 4-8: Numbers of IFN-γ secreting T cells following i.m. pcDNA3.1/core vaccination

The number of spots per well produced by IFN-γ secreting T cells are shown. Cells from BALB/c mice immunised with pcDNA3.1/core (●), pcDNA3.1 alone (o) and from naïve animals (□) are shown. T cells were recovered 10 days after vaccination and restimulated for 5 days prior to the ELISPOT assay. SFC represent the mean number of spots counted in duplicate wells containing 50μ/ml HBcAg peptide $CD8^+$ p87-95.

4.5 Conclusion

As a result of work described in this chapter, it was felt that appropriately sensitive and discriminating methods had been developed for studying the cellular and humoral immune response to HBcAg. These assays therefore form the foundation for subsequent studies, which were carried out to compare the relative response to different vaccination strategies described in the remainder of this thesis.

5 RESULTS – GENERATION OF AN IMMUNE RESPONSE FOLLOWING SYSTEMIC DELIVERY OF A DNA VACCINE ENCODING THE HBCAG

5.1 Chapter Aims

The aim of this chapter was to examine the humoral and cellular HBcAg-specific responses generated following vaccination with the naked plasmid vaccine pcDNA3.1/core. The effects of delivering this vaccine either directly into the skin using a gene gun or into the muscle by injection were observed. In addition, consideration is given to the subsequent enhancement of these responses by i.m. delivery of a purified protein boost.

5.2 Introduction

It has previously been shown that the route of immunisation for DNA vaccines can significantly affect the magnitude and quality of the immune response generated (see Introduction section 1.5.4.3). In general, DNA vaccines delivered intramuscularly (i.m.) require high amounts of DNA to stimulate measurable antibody and cellular responses. In the literature, these appear to be dominated by the stimulation of helper $CD4^+$ T cells of the Th1 phenotype. In contrast, when DNA is given intradermally using a gene gun, relatively low doses of the vaccine are required to generate equivalent levels of immunity, and the response is dominated by $CD4^+$ T cells of the Th2 phenotype. However, most studies to date have examined soluble, non-particulate antigens. This chapter describes the impact of a

DNA vaccine based on a large particulate antigen. This work provides a strong foundation on which the subsequent impact of additional heterologous boost vaccinations can be considered.

To determine the effect of the HBcAg-encoding DNA vaccine pcDNA3.1/core in stimulating various aspects of the immune response, mice were initially given 2 doses of the vaccine either by intramuscular (i.m.) injection or via the skin using a gene gun. To avoid any bias of the inbred mouse strains towards a particular type of immune response, experiments were carried out in both BALB/c and C57/Bl6 mice. The subsequent humoral and cellular responses that were measured are described below and are considered based on the route of immunisation, with immunity to i.m. vaccination examined in the first instance.

5.3 Humoral Responses to pcDNA3.1/core Following Intramuscular Vaccination

In these experiments, C57/Bl6 mice (H2-b MHC haplotype) were immunised on days 1 and 8 with 100μg of pcDNA3.1/core i.m. Control animals were similarly immunised with 100μg of pcDNA3.1 vector alone to differentiate between responses due to the expressed vaccine antigen and the plasmid backbone. A third group of mice were given two doses of 1μg HBcAg protein i.m. at the same times, to act as a positive control. To monitor the generation of HBcAg specific antibodies, sample bleeds were taken 14 and 31 days following the second vaccination (see Figure 5-1).

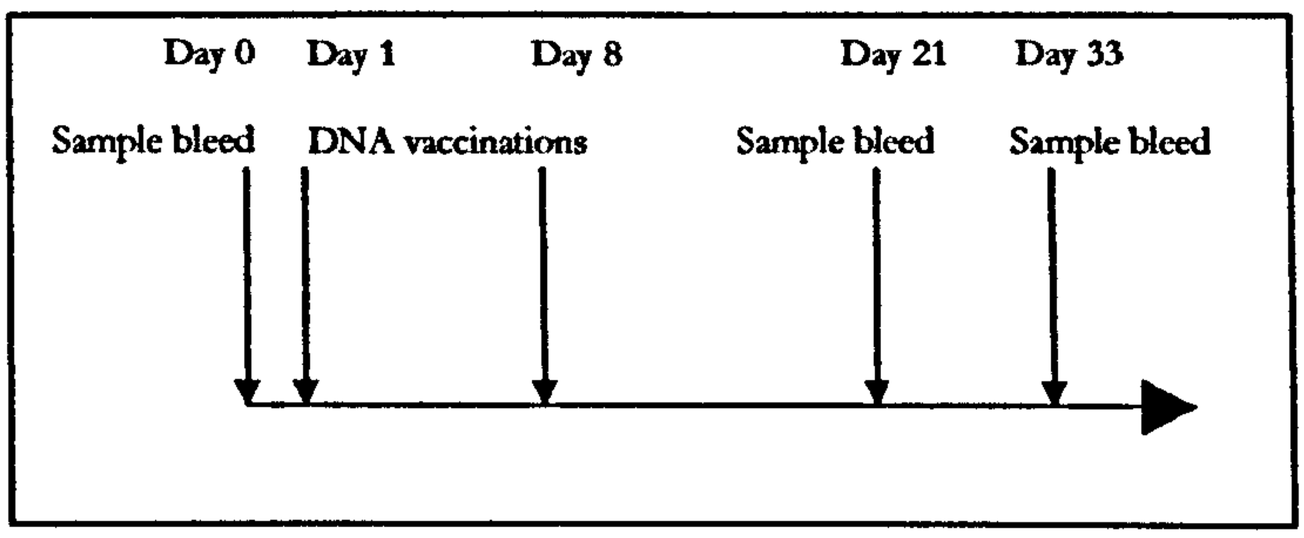


Figure 5-1: Immunisation regimen for mice vaccinated i.m. with the DNA and protein vaccine formulations.

This figure shows the timing of vaccine delivery to the mice and quantification of anti-HBcAg response by assessment of tail sample bleeds taken from each mouse.

HBcAg specific humoral responses were measured by ELISA and are shown in Figure 5-2. In brief, this figure shows that although no detectable responses were observed prior to immunisation (Figure 5-2A), 14 days after the second vaccination, all mice immunised with pcDNA3.1/core had developed low but detectable antibody responses in the sera (Figure 5-2B). The titres generated are approximately ten times lower than those observed in mice immunised with the purified protein. These data indicate the strong immunogenicity of the HBcAg and are consistent with results described elsewhere (Bohm *et al.* 1996). These responses do not appear to improve with time as calculated titres of HBcAg specific antibody had not changed significantly in any group by 39 days following immunisation (Figure 5-2C).

To confirm that the MHC haplotype of the mice does not affect the humoral response generated, the experiments were repeated in BALB/c mice (H2-d haplotype). The results from these experiments are given in Figure 5-3. These confirmed the previous data generated in the C57/Bl6 mice and show that low levels of HBcAg specific antibodies are detectable in all mice immunised with pcDNA3.1/core 14 days after immunisation. No such responses are observed in animals immunised with the vector alone. These responses are lower than those observed in mice immunised with the purified HBcAg material and do not improve over time (Figure 5-3C). These data demonstrated that the DNA vaccine construct was able to stimulate equivalent humoral HBcAg specific responses in two mouse strains of different MHC haplotype.

In order to more fully define the nature of the immune response generated by the DNA vaccine, the pattern of antigen specific IgG isotypes in the sera was also analysed. T helper cell responses can be broadly divided into Th1 or Th2, depending on the cytokines secreted by individual Th cells. These cytokines drive

the production of specific sub-types of immunoglobulin G (IgG), therefore the nature of the T helper cell response can be determined by examining the levels these subtypes. In mice, IgG1 is indicative of a Th2 $CD4^+$ response and IgG2a indicates Th1 (Abbas *et al.* 1996), see Introduction section 1.4.2.2, $CD4^+$ T cell Responses).

Figure 5-4 show the titres of HBcAg-specific IgG subtypes in the sera of the two mouse strains at day 39, after immunisation on days 1 and 8 with pcDNA3.1/core, control DNA or 1μg purified HBcAg. Interestingly, none of the mice immunised with either protein or pcDNA3.1/core show any specific IgG1 production, instead the dominant antibody was IgG2a, although the titres measured varied considerably. This indicates the development of a Th1 response to HBcAg whether vaccination is DNA or protein based.

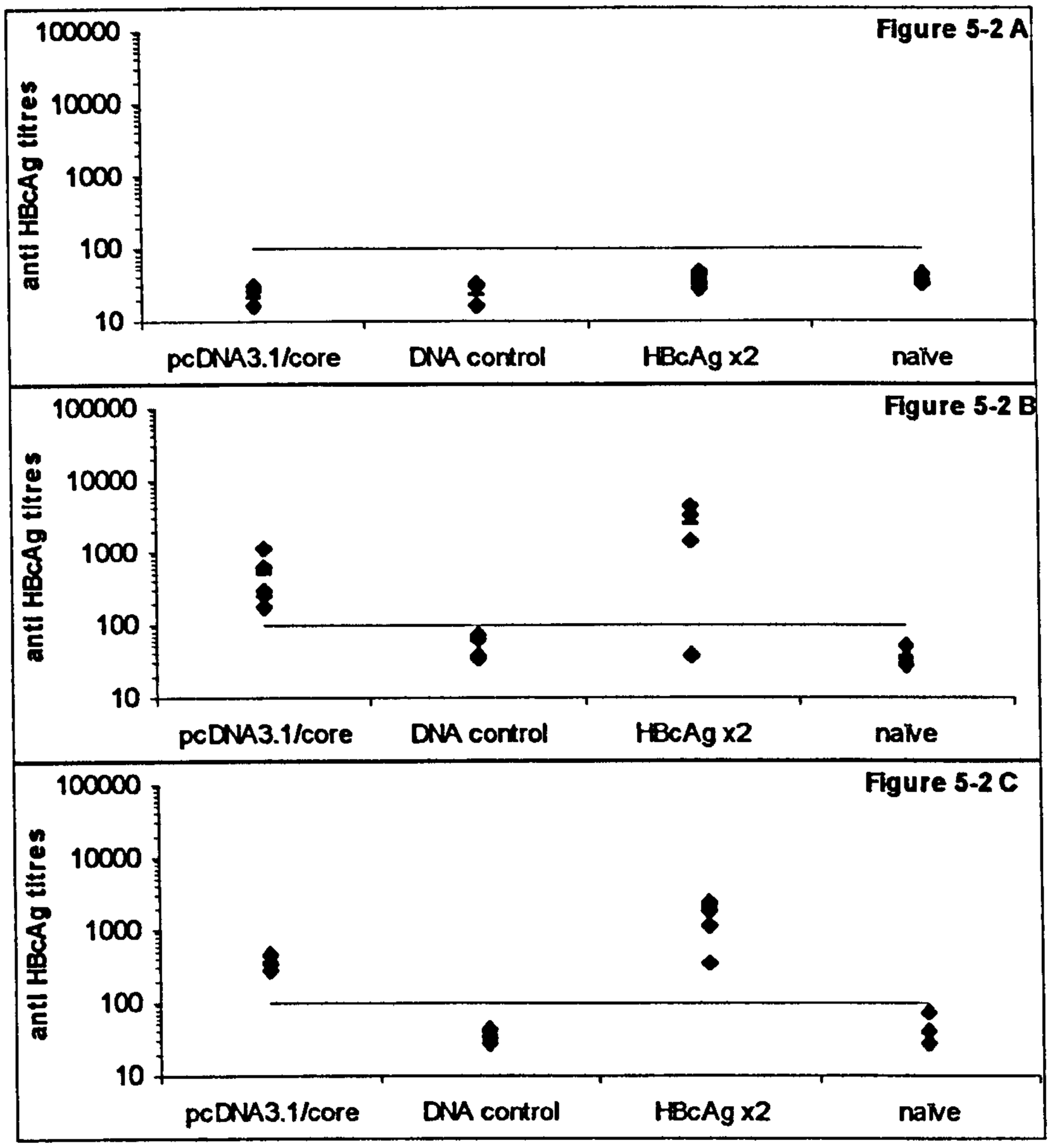


Figure 5-2: i.m. DNA vaccination in C57/Bl6 mice; development of HBcAg-specific serum antibody

Groups of 5 C57/Bl6 mice were immunised i.m. with either 100µg DNA (pcDNA3.1/core and DNA control groups) or 1µg HBcAg protein alone (HBcAg x2 group), on days 1 and 8 of the experiment. Figure 5-2A shows the titres of HBcAg specific immunoglobulin from all mice prior to vaccination, Figure 5-2B at 14 days after the second vaccination and Figure 5-2C shows titres at 31 days after vaccination. HBcAg-specific titres are represented by ◆, groups means by horizontal bars.

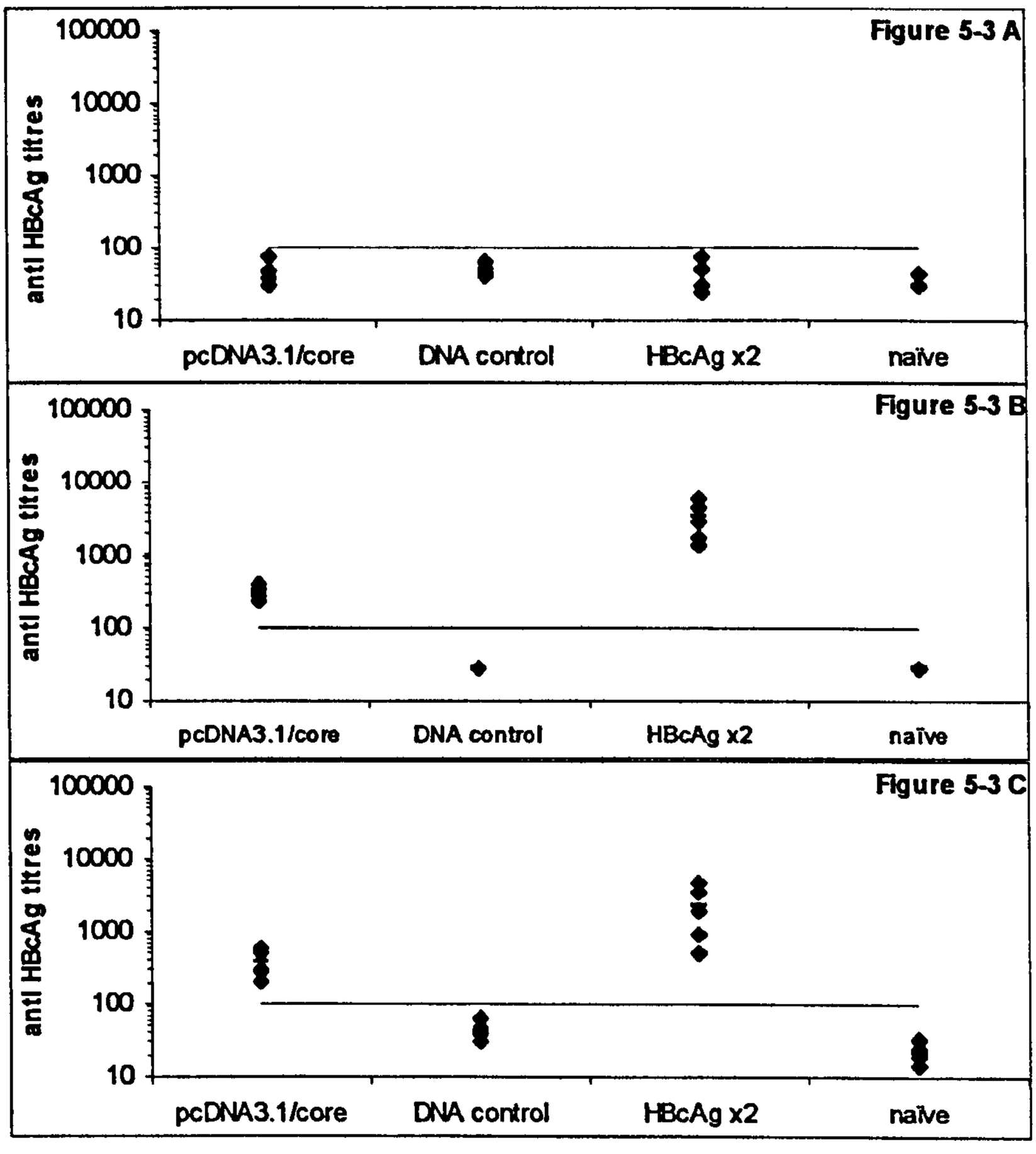


Figure 5-3: i.m. DNA vaccination in BALB/c mice; development of HBcAg-specific serum antibody

Groups of 5 BALB/c mice were immunised i.m. with either 100μg DNA (pcDNA3.1/core and DNA control groups) or 1μg HBcAg protein alone (HBcAg x2 group) on days 1 and 8 of the experiment. Figure 5-3A shows the titres of HBcAg specific immunoglobulin from all mice prior to vaccination, Figure 5-3B at 14 days after the second vaccination and Figure 5-3C shows titres at 31 days after vaccination. HBcAg-specific titres are represented by ◆, groups means by horizontal bars.

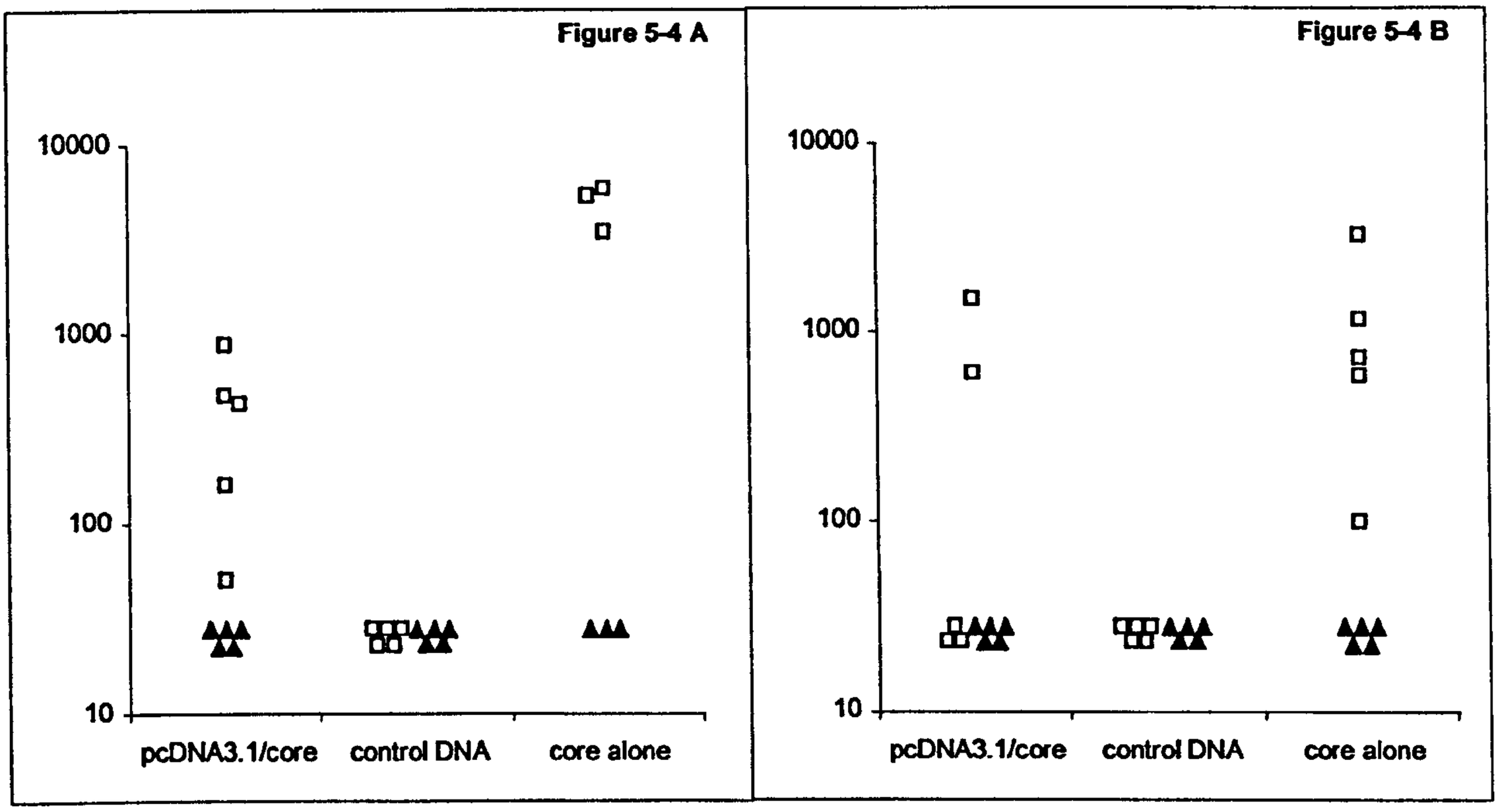


Figure 5-4: Measurement of antibody subtypes in the sera of mice vaccinated with pcDNA3.1/core i.m. or purified HBcAg

BALB/c (Figure 5-4A) and C57/Bl6 (Figure 5-4B) mice were immunised with 100μg pcDNA3.1/core or 1μg HBcAg i.m. on day 1 and day 8. Immunoglobulin subtypes were examined on day 39. Calculated specific anti-HBcAg titres are shown. ▲ represent IgG1 titres for individual mice, □ represent IgG2a titres.

5.4 Cellular Responses to pcDNA3.1/core Following Intramuscular Vaccination of C57/Bl6 mice

5.4.1 $CD4^+$ T Cell Responses

To determine the impact of vaccination on HBcAg specific $CD4^+$ cells, mice were immunised as described in Figure 5-1 and $CD4^+$ T cells purified from spleens and draining lymph nodes (popliteal and inguinal) 14 days after the second vaccine dose. Proliferation was measured by β-emission of these cells following incorporation of ^{3}H-thymidine in culture (Materials and Methods section 2.5.4 – 2.5.5). Unfortunately, despite several attempts using optimised protocols no HBcAg-specific proliferation could be measured in any of the mice. This may have been due to a genuine lack of response in the animals, or problems with sensitivity of the assay (see Chapter 4 for details of the assay development). Another possibility was that there were only low numbers of primed cells at the time point studied. To see if this was the case, $CD4^+$ responses were measured using an alternative vaccination protocol. In this experiment, mice were immunised on days 1 and 28 and T cell proliferation examined on day 56 of the experiment. It was thought that the greater time interval between immunisations and killing would allow a longer period of antigen expression by the cells leading to stimulation of a greater number of naïve T cells.

The results of this experiment are shown in Figure 5-5 and confirm that antigen-specific T cell proliferation can be observed in the spleens of these mice. These responses were dramatically higher than those observed in cells from naïve mice. In contrast no detectable response was observed in the cells taken from the popliteal lymph nodes (Figure 5-5B). This suggests that the antigen specific cells

have matured and trafficked to the spleen at this time point. This longer-term vaccination regime was not adopted for general use since these experiments were conducted toward the end of the experimental work for this thesis and too late to be incorporated into the general scheme of work.

5.4.2 $CD8^+$ T Cell Responses

To measure HBcAg specific $CD8^+$ T cell responses, mice were given a single dose of 100μg pcDNA3.1/core and killed 10 days later. Splenic cells from these mice were isolated and restimulated *ex vivo* for 5 days with P815/c target cells, as described in Materials and Methods section 2.5.6. The killing capacity of these restimulated cells is shown in Figure 5-6. These data clearly demonstrate the induction of antigen-specific CTLs following vaccination with a single 100μg i.m. dose of the pcDNA3.1/core vector. At effector : target cell ratios of 50:1 and 25:1 this killing response is approximately 25% greater than that observed with the equivalent number of cells taken from naïve animals or those immunised with the vector alone.

An alternative method of evaluating the CTL response is to measure the number of IFN-γ secreting cells in an ELISPOT assay (Materials and Methods section 2.5.7). Cells taken from mice immunised twice with pcDNA3.1/core, as before, yielded the results shown in Figure 5-7.

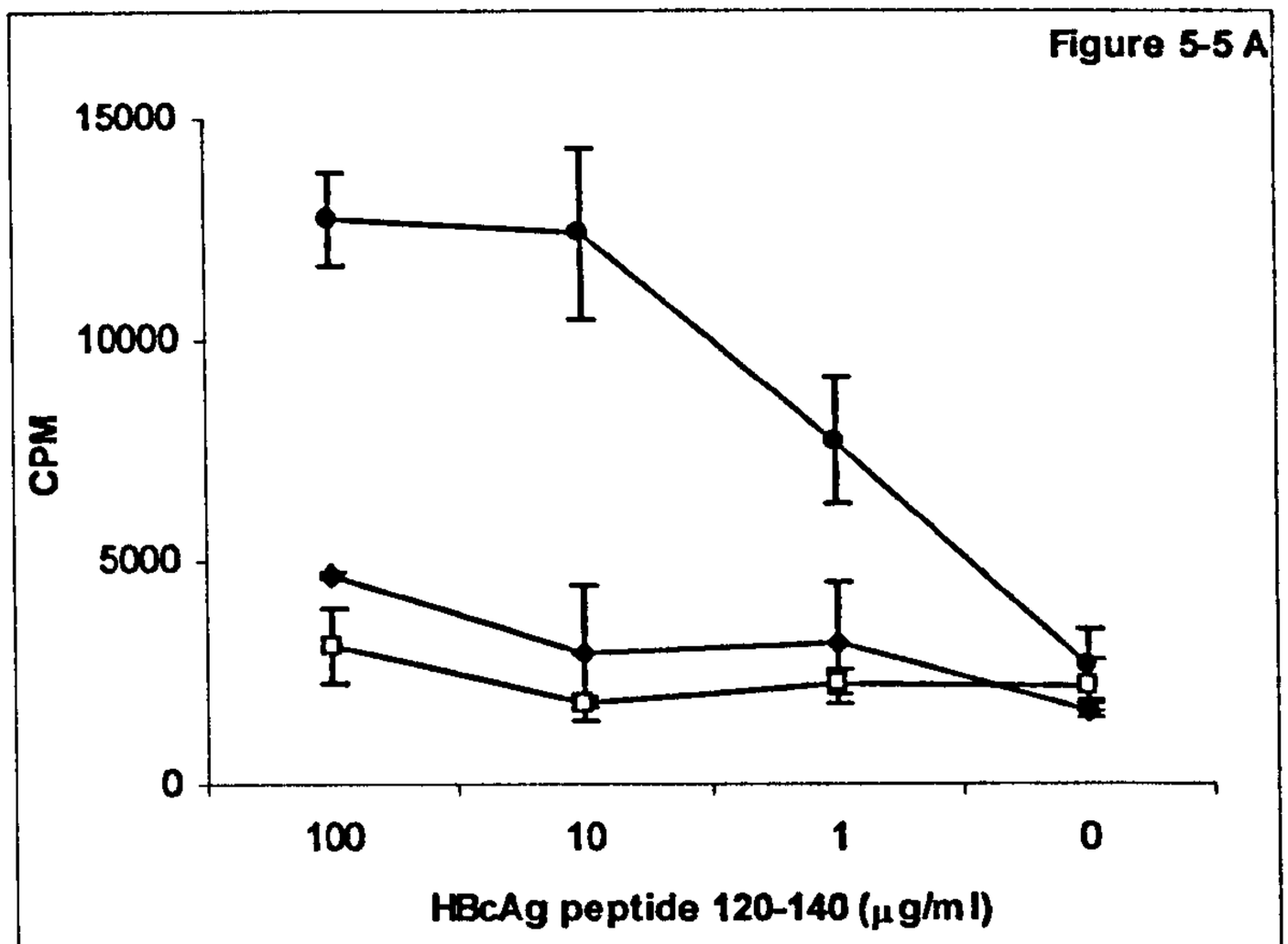
The number of spot forming cells (SFC) are higher in the pcDNA3.1/core group than the DNA control and naïve groups, at both the 10:1 and 5:1 E:APC ratios, irrespective of the quantity of restimulation antigen used in the assay (the results shown are from cultures stimulated with 50μg/ml HBcAg peptide (p93-100). This confirms that vaccination with pcDNA3.1/core activated $CD8^+$ T cells and

shows that $CD8^+$ T cells from naïve animals were not activated to produce IFN-γ during the assay.

These results are in line with and expand upon previous findings showing that i.m. DNA vaccination can induce an anti-HBcAg specific CTL killing (Kuhrober *et al.* 1996).

5.5 Summary of Results

From these initial *in vivo* experiments, it was clear that the plasmid pcDNA3.1/core was able to stimulate humoral and cellular immune responses following i.m. administration. The results shown here represent typical data from several experiments and did not appear to be affected by the haplotype of the mouse strain used. Therefore unless stated, all later experiments were carried out in C57/Bl6 mice only. These data confirm similar observations in the literature that i.m. DNA immunisation induces strong antigen specific $CD8^+$ responses and low but detectable humoral responses. It was not possible to detect a measurable antigen specific $CD4^+$ response without increasing the time between immunisations and the measurement of proliferation. This may be due to the limit of sensitivity of the assay employed or an only gradual activation of such cells because of long-term, low level antigen expression.



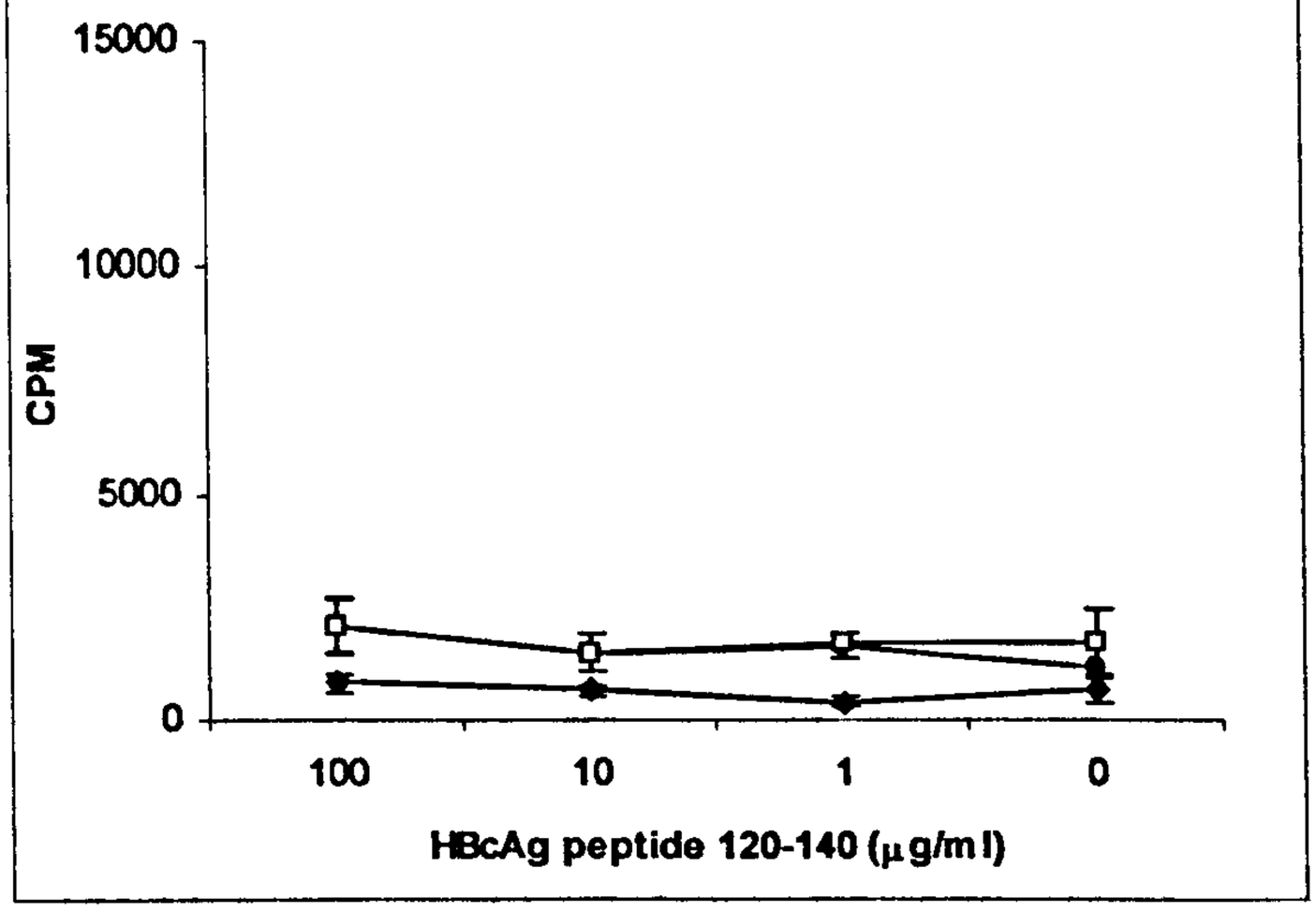


Figure 5-5: Measurement of CD4⁺ T cell proliferation *in vitro* using cells from mice immunised i.m. with pcDNA3.1/core

C57/Bl6 mice were immunised with 100µg of DNA i.m. on days 1 and 8 of the experiment, or 1µg HBcAg alone on day 8, and sacrificed on day 56. Spleen (Figure 5-5A) and popliteal and inguinal lymph node cells (Figure 5-5B) were recovered from mice immunised with pcDNA3.1/core (●) or naïve mice (□). Control mice received only the 1µg dose of HBcAg i.m. (◆). The proliferation of cells from pooled mice was measured by β-emission from cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of peptide concentrations. Assay described in full in materials and methods sections 2.5.2 – 2.5.4. Mean CPM from triplicate culture wells are shown ± 1SD.

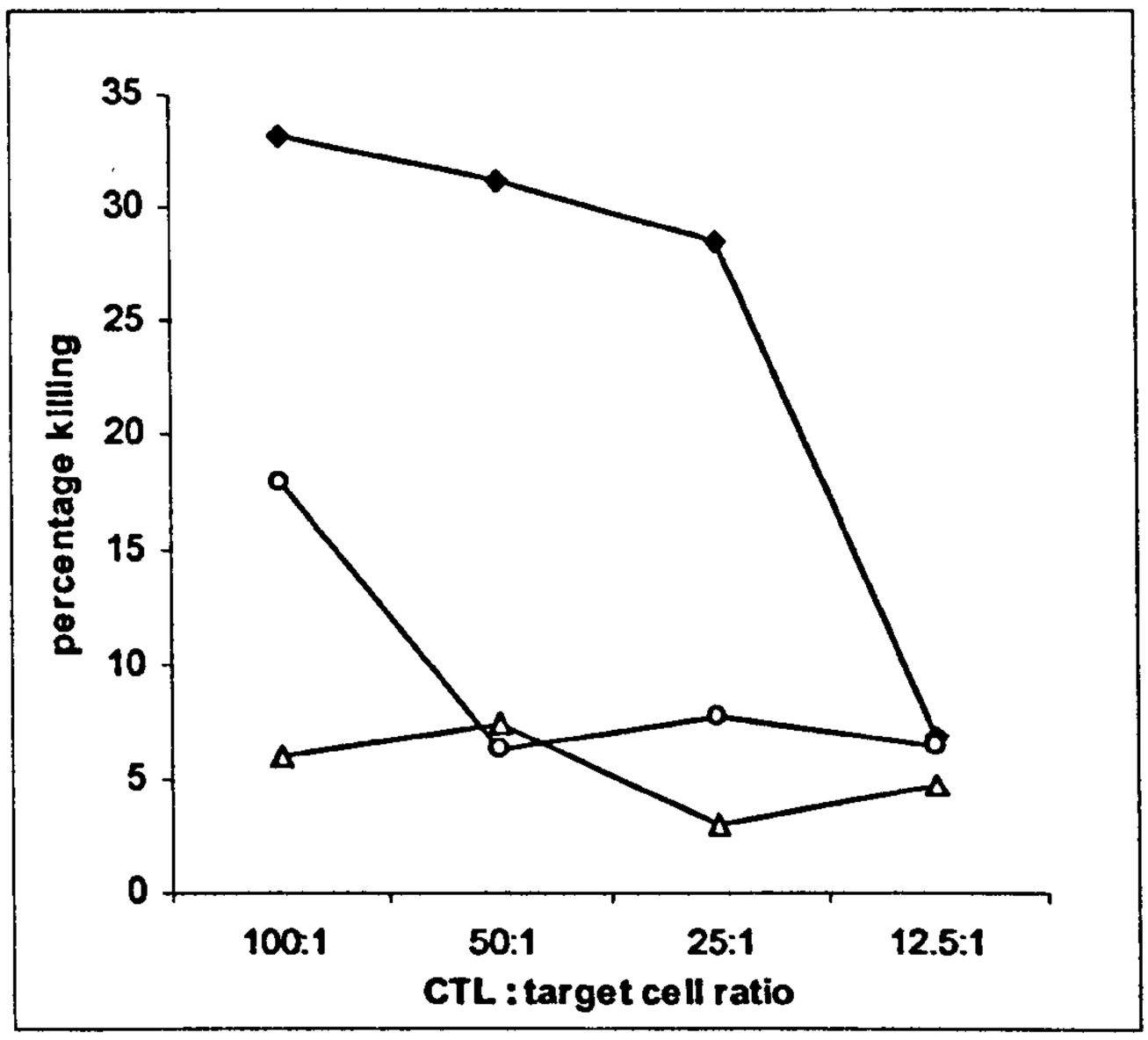


Figure 5-6: Specific killing by spleen cells of DNA immunised mice

Percentage specific killing of p815/c target cells by spleen cells of C57/Bl6 mice immunised i.m. with 100µg pcDNA3.1/core. Groups of 5 mice were immunised with 100µg pcDNA3.1/core (◆) or pcDNA3.1 (○) or were left unimmunised (Δ) and were sacrificed 10 days after the final vaccination. The spleen cells were restimulated *ex vivo* for 5 days with p815c target cells (5 target cells per 100 splenocytes) at 37°C, 5% CO_2. The cells were then added to fresh, ^{51}Cr labelled target cells at different ratios for a 4 hour killing assay, as described in full in section 2.5.5.

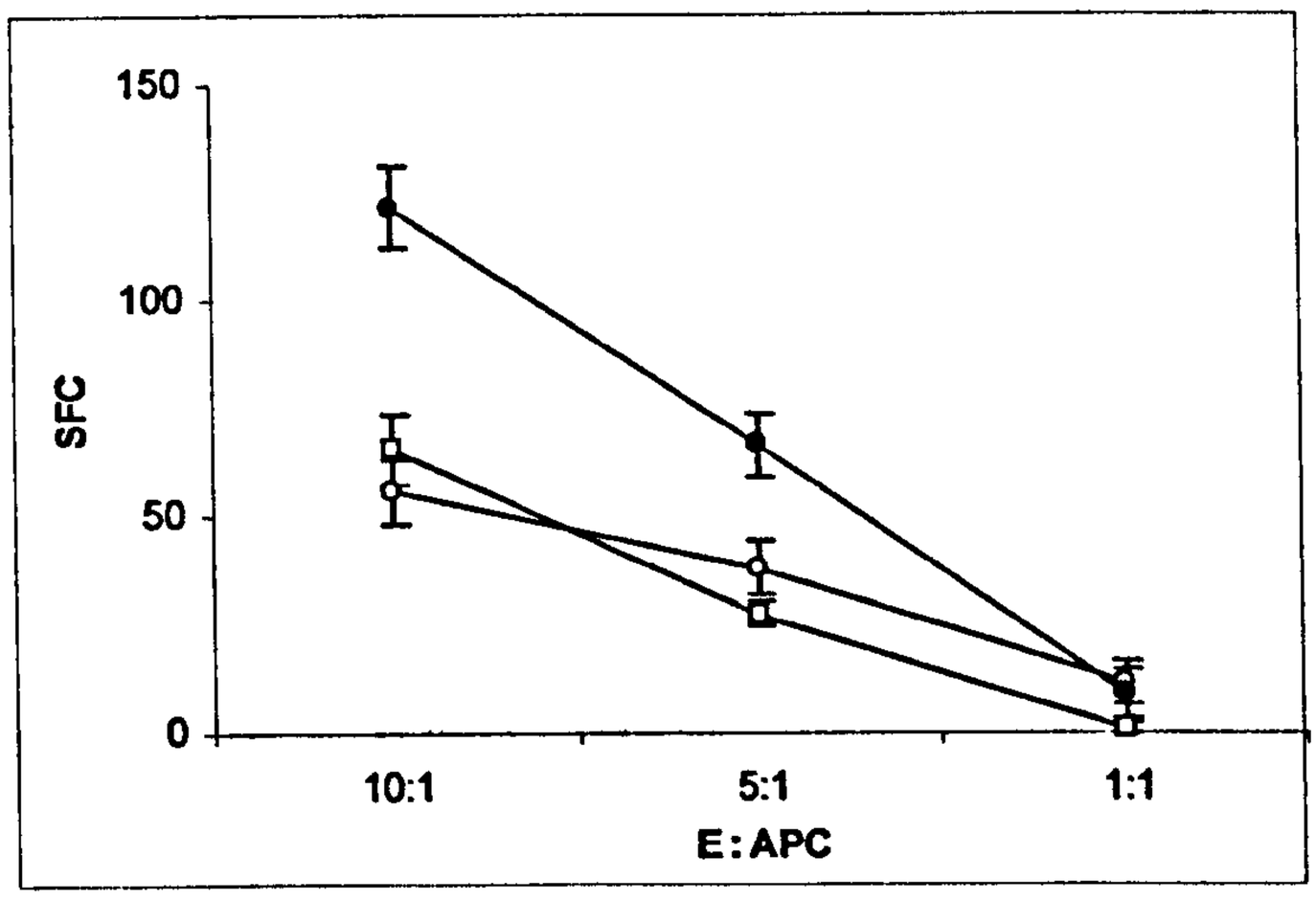


Figure 5-7: CD8⁺ T cell activation in DNA vaccinated mice measured by IFN-γ ELISPOT

C57/Bl6 mice were immunised on day 1 and 8 with 100μg of pcDNA3.1/core (●), pcDNA3.1 (o) or remained naïve (□), and sacrificed on day 15. Cells were incubated in a 5 day restimulation assay and IFN-γ secreting cells quantified by ELISPOT at different effector : APC ratios. Cells were incubated with 50μg/ml HBcAg p93-100 peptide during the assay. Error bars represent the standard deviation of three SFC counts for each well.

5.6 Enhancement of Immunity Through Prime-Boost Vaccination Strategies

For a DNA vaccine candidate to be of maximal potential use, strong cellular and humoral responses should be activated following immunisation. Therefore in an attempt to augment the $CD4^+$ and antibody responses to HBcAg (and to provide both types of response simultaneously) the potential for combining pcDNA3.1/core vaccination with different boosts was investigated. The remainder of this chapter describes the investigations into whether immunity to the HBcAg generated by the i.m. DNA vaccine could be enhanced by boosting with a small quantity of purified HBcAg protein, also administered by the i.m. route. Figure 5-8 shows the revised vaccination schedule and the timing of this boost immunisation.

5.7 Humoral Responses Following i.m. DNA Prime and Purified i.m. HBcAg Boost

For these experiments, animals were primed with pcDNA3.1/core, as described previously, on days 1 and 8, and then boosted with 1μg HBcAg on day 40 of the experiment (Figure 5-8). As before, to determine the response to the DNA vector backbone, control mice were immunised with the vector alone and then boosted with purified HBcAg as described above. To allow comparison with mice given the purified protein alone, some mice were injected with the purified protein rather than DNA (HBcAg x3), at the same timepoints. Finally, to determine the impact of the boost alone, previously naïve mice were given a single 1μg dose of HBcAg (x1) on day 40. To confirm no differences were observed using mice of

different haplotype, these experiments were performed in both BALB/c and C57/Bl6 mice.

The effect of the boost is shown in Figure 5-9 (C57/Bl6 mice) and Figure 5-10 (BALB/c mice). In both strains, those animals primed with pcDNA3.1/core show a greater than ten-fold increase in mean titres. The increase in the BALB/c mice pcDNA3.1/core group following the boost is significant (Student's t-test, $p=0.042$), however that observed in the equivalent C57/Bl6 group is not ($p=0.079$), although it is consistent between experiments.

It is clear that there is a much greater increase in HBcAg-specific antibody titres in these groups than is observed in either the mice primed with the vector alone or those animals receiving the boost immunisation alone. In contrast, there was little change in the antibody titres observed in mice previously immunised with 2 doses of HBcAg. This is possibly because the maximal antibody response had already been achieved in these animals. Interestingly, the mean titre of the group immunised with pcDNA3.1/core HBcAg and boosted with protein was approximately equivalent to that observed at the final time-point of those given the purified antigen alone, despite low titres measured prior to the boost.

5.7.1 Subtype Responses

To determine the impact of the boost on the nature of the $CD4^+$ Th response in these animals, the presence of different IgG isotypes within the HBcAg-specific serum antibody response were examined. The titres are shown in Figure 5-11. On boosting, titres of IgG2a increased in all mice primed with pcDNA3.1/core, although there was still a broad range of titres in identically immunised animals. As before, in C57/Bl6 mice, there was no measurable IgG1

production. In the BALB/c mice, one out of 5 animals showed a low level of IgG1 production, whilst the others showed no response. This combination of responses indicates the maintenance of the Th1 dominated response seen following DNA vaccination alone.

5.8 Cellular Responses Following i.m. DNA Prime and Purified i.m. HBcAg Boost Vaccination

5.8.1 $CD4^+$ T Cell Proliferation

Splenocytes taken from mice immunised with two doses of pcDNA3.1/core and boosted with HBcAg protein were cultured in proliferation assays, the results of which are shown in Figure 5-12. In contrast to earlier experiments when no detectable $CD4^+$ responses could be observed in animals immunised with pcDNA3.1/core alone (in a short-term vaccination regime), animals primed with pcDNA3.1/core and boosted with purified HBcAg showed strong antigen specific proliferation. These responses were equivalent in magnitude to those observed in animals that received three doses of the protein. Interestingly, a single dose of the HBcAg was not sufficient to cause proliferation in previously naïve mice, but did result in low level proliferation in control DNA-primed mice. This may have been due to the non-specific immuno-potentiating activity of the CpG motifs in the control DNA.

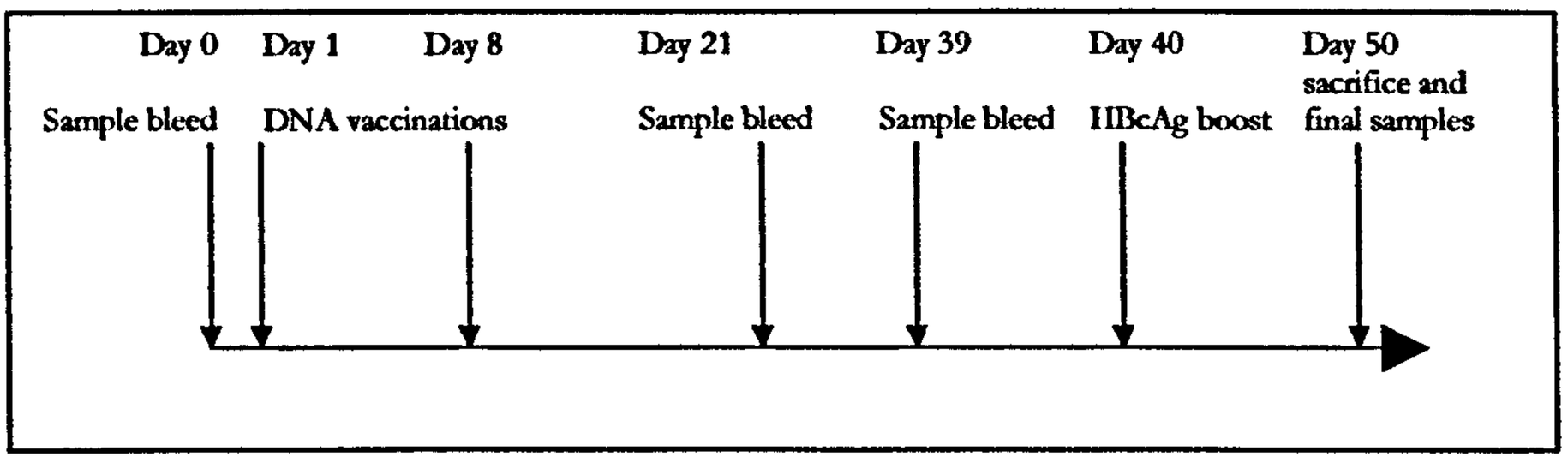


Figure 5-8: Revised vaccination regime for pcDNA3.1/core immunisation including a subsequent heterologous boost with i.m. recombinant HBcAg

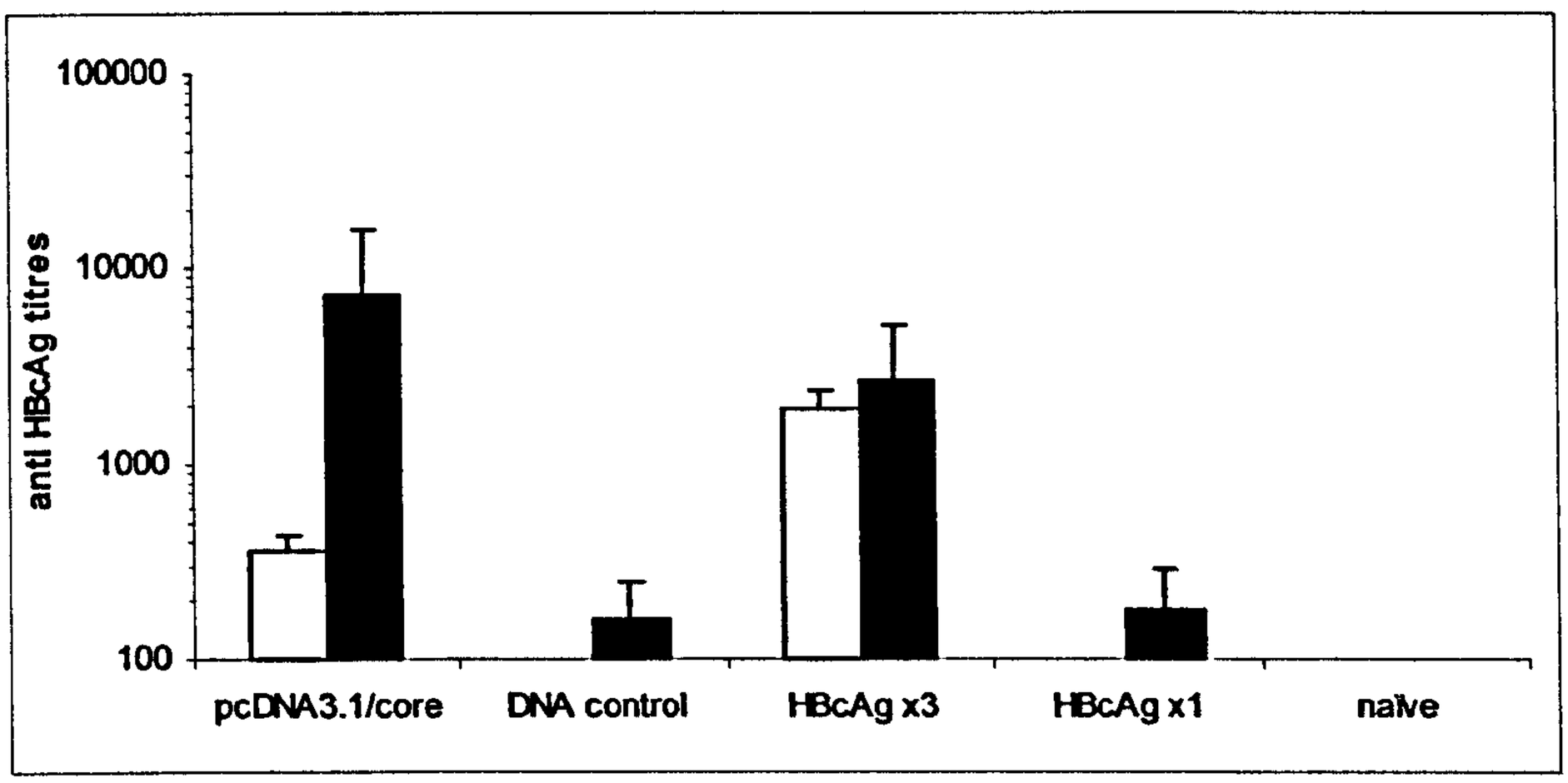


Figure 5-9: Impact of i.m. boost with purified HBcAg on HBcAg-specific serum antibody titres

C57/Bl6 mice were immunised as described in Figure 5-8. The graph shows the mean titres of HBcAg-specific antibody calculated from 5 individual animals. Sample bleeds were taken 1 day prior to the boost immunisation (□) or 10 days following the boost (■). Error bars represent the standard deviation from the mean titre of each group.

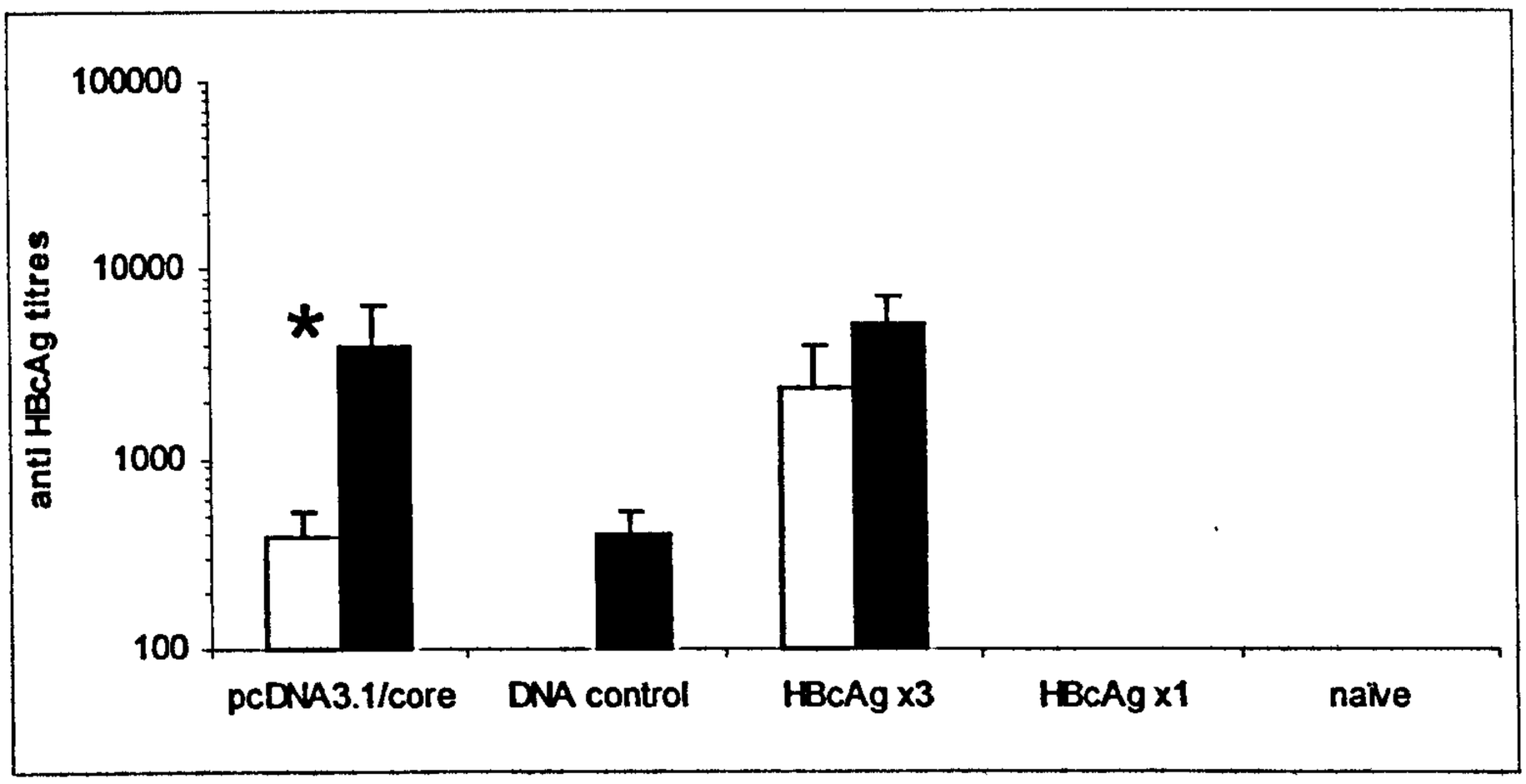


Figure 5-10: Impact of i.m. boost with purified HBcAg on HBcAg-specific serum antibody titres

BALB/c mice were immunised as described in Figure 5-8. The graph shows the mean titres of HBcAg-specific antibody calculated from 5 individual animals. Sample bleeds were taken 1 day prior to the boost immunisation (□) or 10 days following the boost (■). Error bars represent the standard deviation from the mean titre of each group. * indicates a significant difference between titres in the pcDNA3.1/core group following the HBcAg boost.

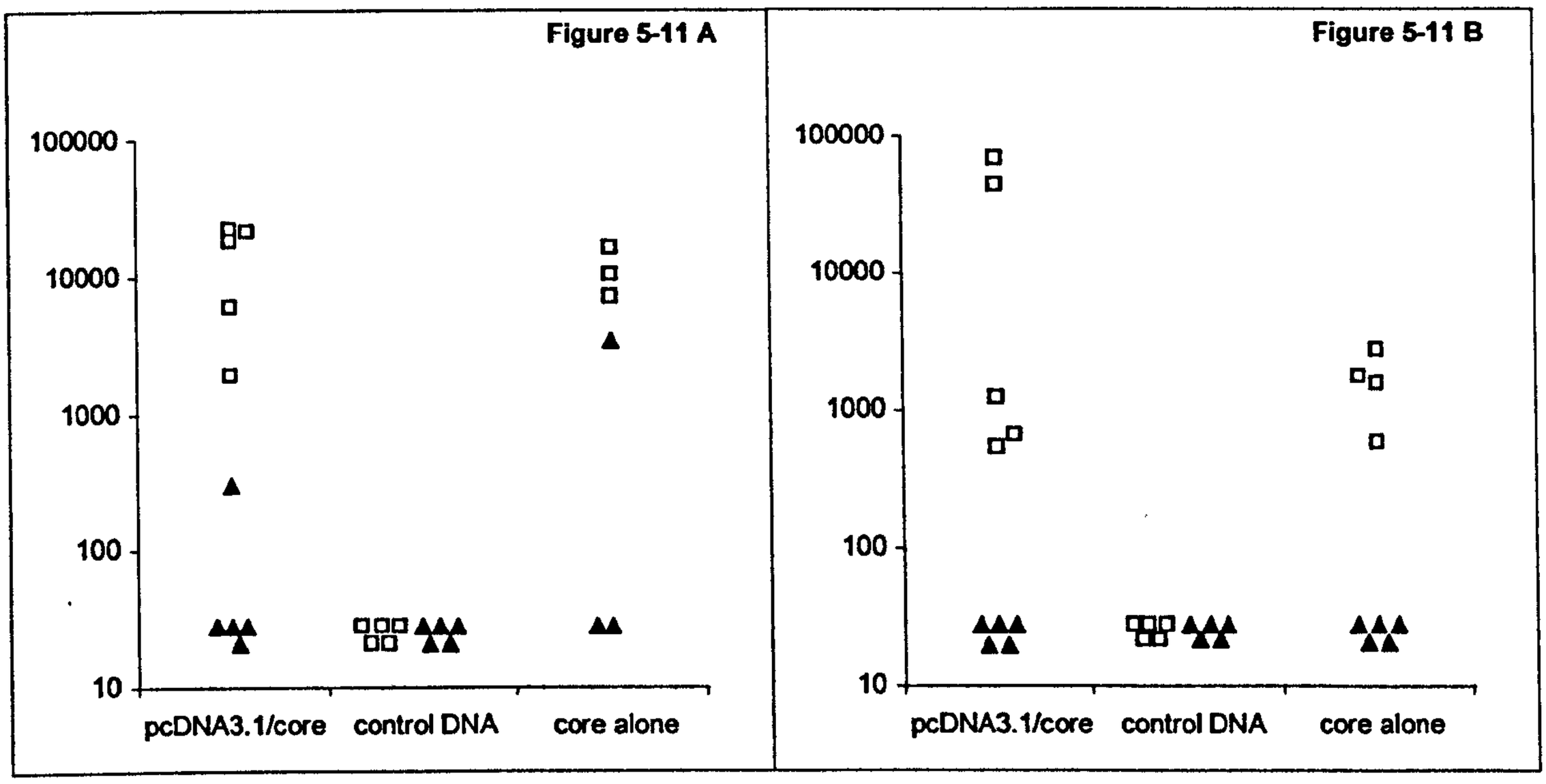


Figure 5-11: Measurement of antibody subtypes in the sera of mice vaccinated with pcDNA3.1/core and boosted with purified HBcAg

BALB/c (Figure 5-11A) and C57/Bl6 (Figure 5-11B) mice were immunised with 100μg pcDNA3.1/core or control DNA on days 1 and 8, and boosted with 1μg HBcAg i.m. on day 40 (as described in Figure 5-8). Immunoglobulin subtypes were examined on day 50. Calculated specific anti-HBcAg titres are shown. ▲ represent IgG1 titres for individual mice, □ represent IgG2a titres.

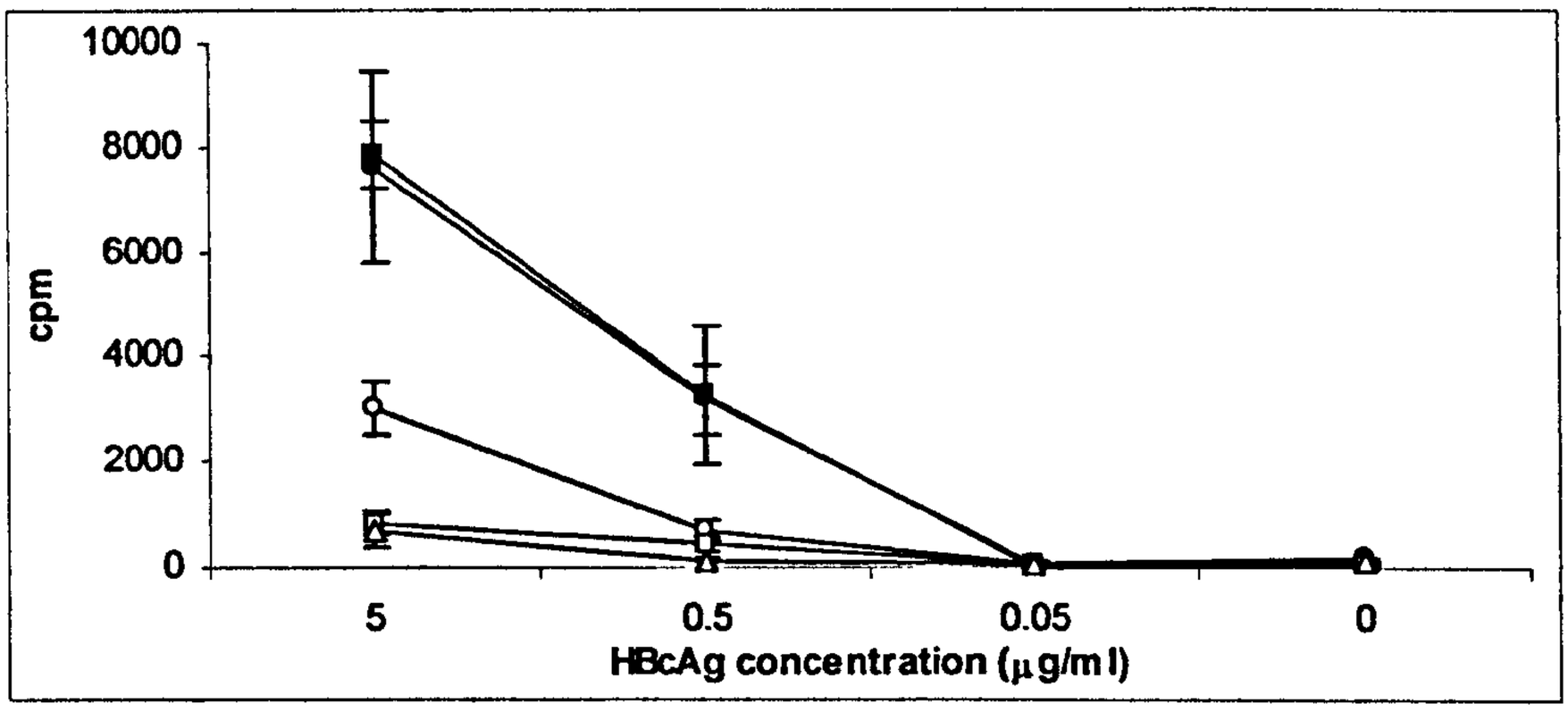


Figure 5-12: Impact of i.m. boost with purified HBcAg on specific proliferation of CD4⁺ T cells from mice primed with pcDNA3.1/core

C57/Bl6 mice were immunised according to the schedule described in Figure 5-8. 100µg pcDNA3.1/core (●) or control DNA (○)was delivered i.m. on days 1 and 8, and mice were boosted on day 40 with 1µg purified HBcAg i.m. Control groups were given three doses of 1µg purified HBcAg at the same timepoints (■) or a single dose on day 40 (□). One group remained untreated (Δ). All mice were sacrificed and splenocytes recovered on day 50. Pooled splenic populations were stimulated *in vitro* and the level of proliferation measured by incorporation of tritiated thymidine. The counts shown are the mean values calculated from triplicate wells of pooled cells stimulated with different concentration of protein. Error bars represent the standard deviation from the mean.

5.8.2 $CD8^+$ T Cell Responses

$CD8^+$ T cell activation was measured by ELISPOT assay, as shown in Figure 5-13. As with DNA vaccination alone, the prime-boost strategy was able to activate greater numbers of cells than when a single dose of HBcAg was given.

5.9 Comparison of the Route of Administration of the DNA Vaccines on Immunity: i.m. Vaccination Versus Gene Gun Delivery

Based on the impressive results achieved by boosting the DNA primed mice with purified antigen, we looked to improve the overall prime boost strategy employed. As i.m. vaccination requires very high amounts of DNA per dose, it is unlikely to be ever considered as suitable for use in humans. Alternative methods such as use of gene gun have therefore been used clinically. However, previous experiments have shown that the use of gene gun can alter significantly the type of immune response primed. The impact of priming animals with DNA by gene gun on subsequent responses following boosting with purified protein was therefore considered.

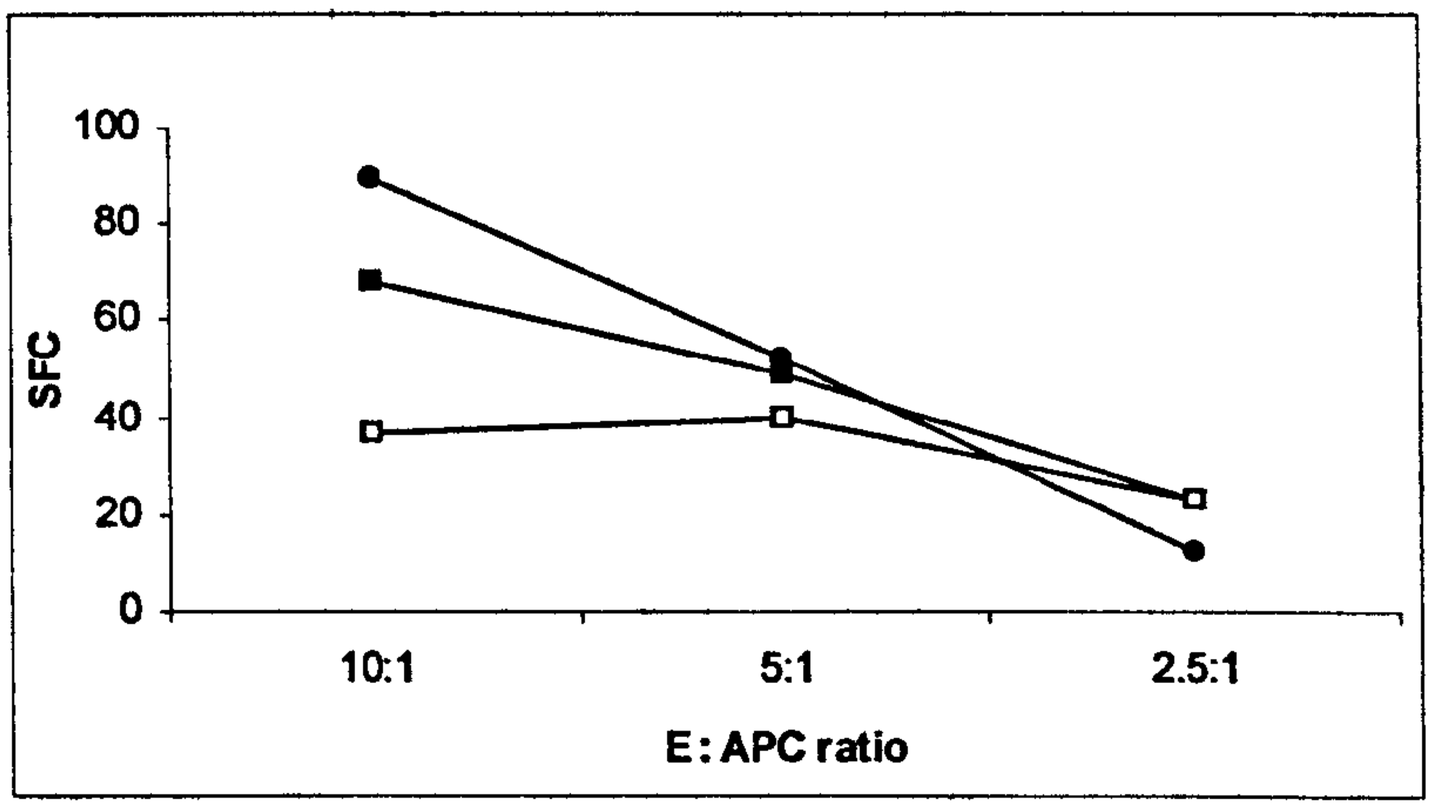


Figure 5-13: Measurement of $CD8^+$ T cell activation in DNA primed, purified HBcAg boosted mice by IFN-γ ELISPOT

Mice were immunised twice with 100μg of pcDNA3.1/core (●) or psDNA3.1 alone (■) and boosted with 1μg HBcAg i.m. before sacrifice 10 days later. One group remained untreated (□). Cells were incubated in a 5 day restimulation assay and IFN-γ secreting cells quantified by ELISPOT at different effector : target cell ratios. Cells were incubated with 200μg/ml HBcAg p93-100.

5.9.1 Humoral Responses

DNA vaccination using a gene gun is fully described in the Introduction (section 1.5.4).

For these experiments, animals were again immunised using the schedule shown in Figure 5-8. Animals given i.m. immunisations were given 100μg of DNA per dose whilst in parallel, animals immunised using the gene gun were given with between 1-2μg per dose (Figure 5-14). Despite the lower dosage, the anti-HBcAg antibody titres calculated were not significantly different to those observed in animals immunised i.m. with pcDNA3.1/core (Figure 5.14B, C). Control DNA did not elicit an antibody response by either route.

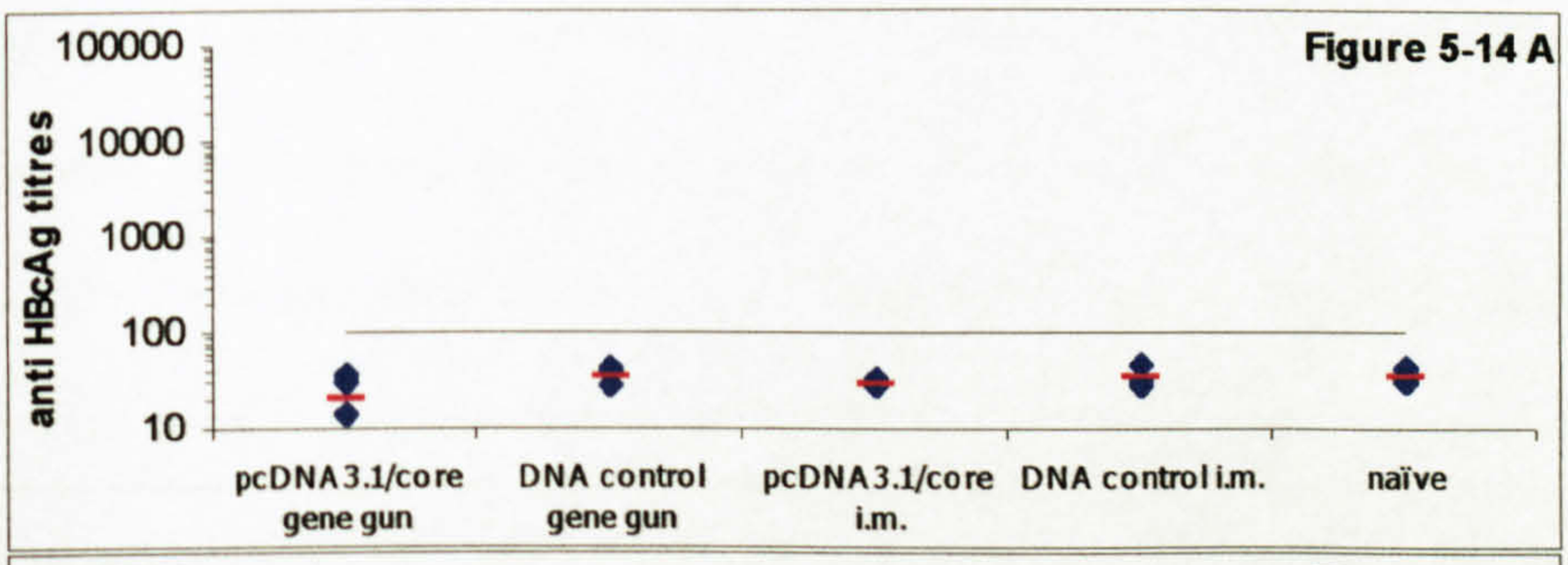
5.9.2 Impact of Purified Protein Boost on Gene Gun-Primed Mice Compared to i.m.-Primed Mice

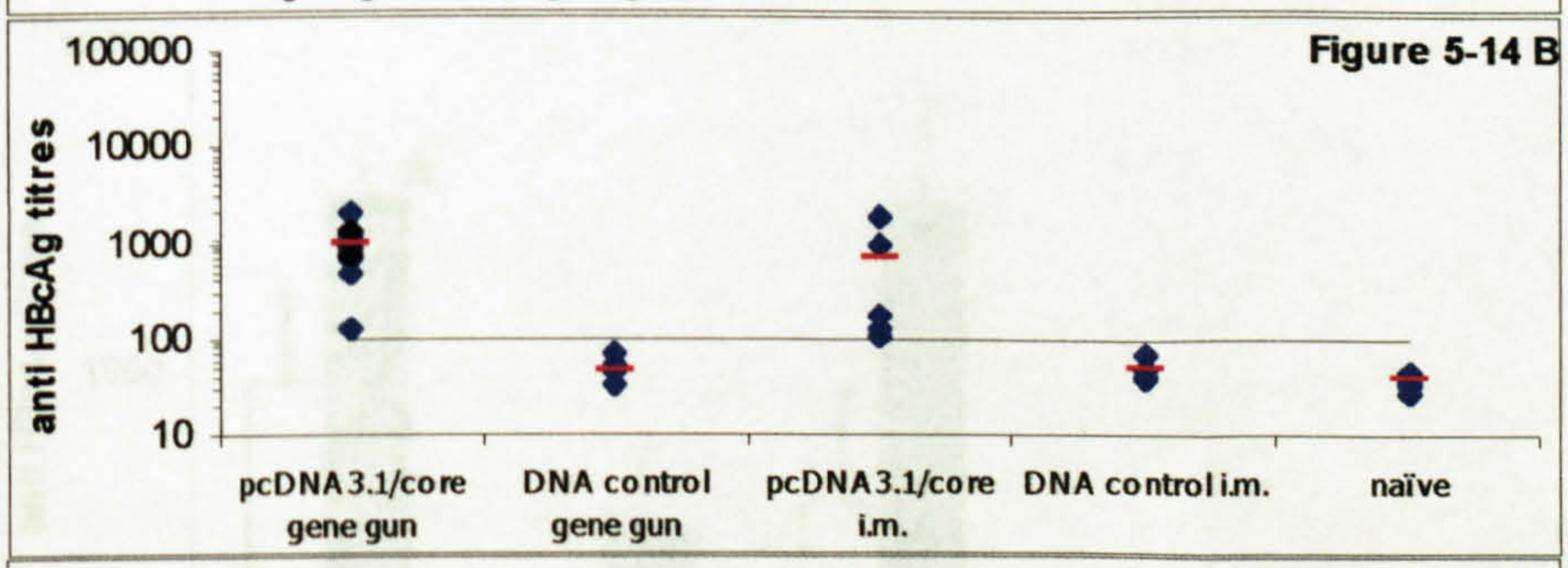
5.9.2.1 Impact of Boost on Humoral Responses

Following boosting of these primed animals with purified protein, titres of antibodies were again enhanced to an approximately equivalent level (Figure 5-15). In contrast, the mice immunised with the control vector i.m. and the naïve animals failed to respond to the antigen alone. Interestingly, the mice given the vector alone by gene gun and then boosted showed a low but measurable response to the antigen. It is unclear why this occurred but may reflect the sensitivity of the immune system to CpG motifs delivered using the intradermal route. Indeed, this may also explain why slightly stronger responses were observed in the gene gun group compared to i.m. vaccinated DNA control mice, (although not statistically significant). Interestingly, animals vaccinated with the gene gun responded more

consistently; with less variation between individual titres observed in this group. For this reason the increase in titres following the boost are highly statistically significant ($p<0.001$) and the more varied titres in the i.m. group following vaccination do not constitute a significant increase, although the observation of an increase was consistent between experiments.

The titres of IgG subtypes 1 and 2a in the serum of mice on day 50 are shown in Figure 5-16. Both gene gun and i.m. immunisation led to the development of HBcAg-specific IgG2a titres. As before, the i.m. DNA immunisation followed by purified HBcAg boost resulted in an IgG2a dominated response, as was the case with animals given control DNA by either gene gun or i.m. routes followed by the i.m. protein boost. In contrast, as well as strong IgG2a titres, immunisation with the gene gun resulted in strong IgG1 titres in most mice. This is indicative of a more Th2-like immune response (Abbas *et al.* 1996) and is consistent with other observations that have indicated that immunisation with gene gun induces a greater Th2 response.





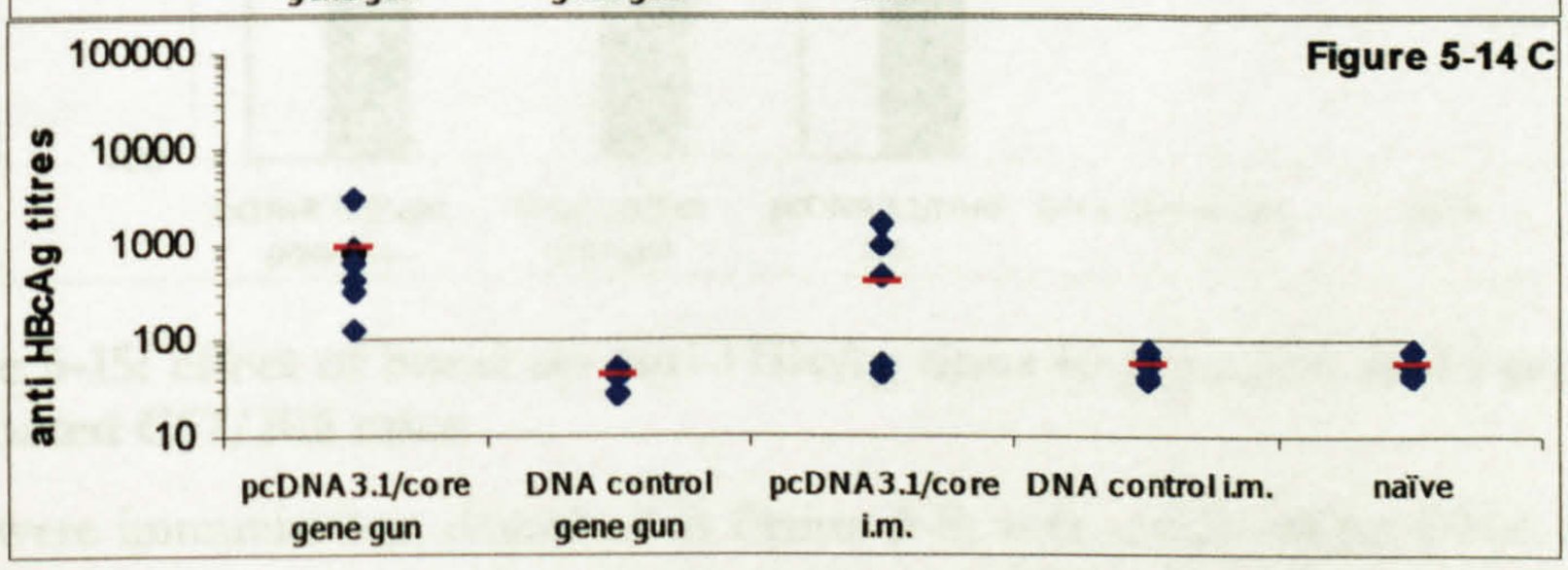


Figure 5-14: Development of antibody titres following DNA vaccination by gene gun or i.m. injection

Groups of C57/Bl6 mice were immunised either i.m. with 100µg pcDNA3.1/core or by gene gun (10 mice by gene gun, 5 in all others) with 1µg of the same plasmid, on days 1 and 8 of the experiment. Anti-HBcAg specific antibodies were measured in blood samples taken prior to the first immunisation (Figure 5-14A), 14 days after the second vaccination (Figure 5-14B) or 31 days after the second vaccination (Figure 5-14C). Titres of individual mice are represented as ◆, the mean value of each group as horizontal bars and the threshold of sensitivity of the assay by the horizontal line.

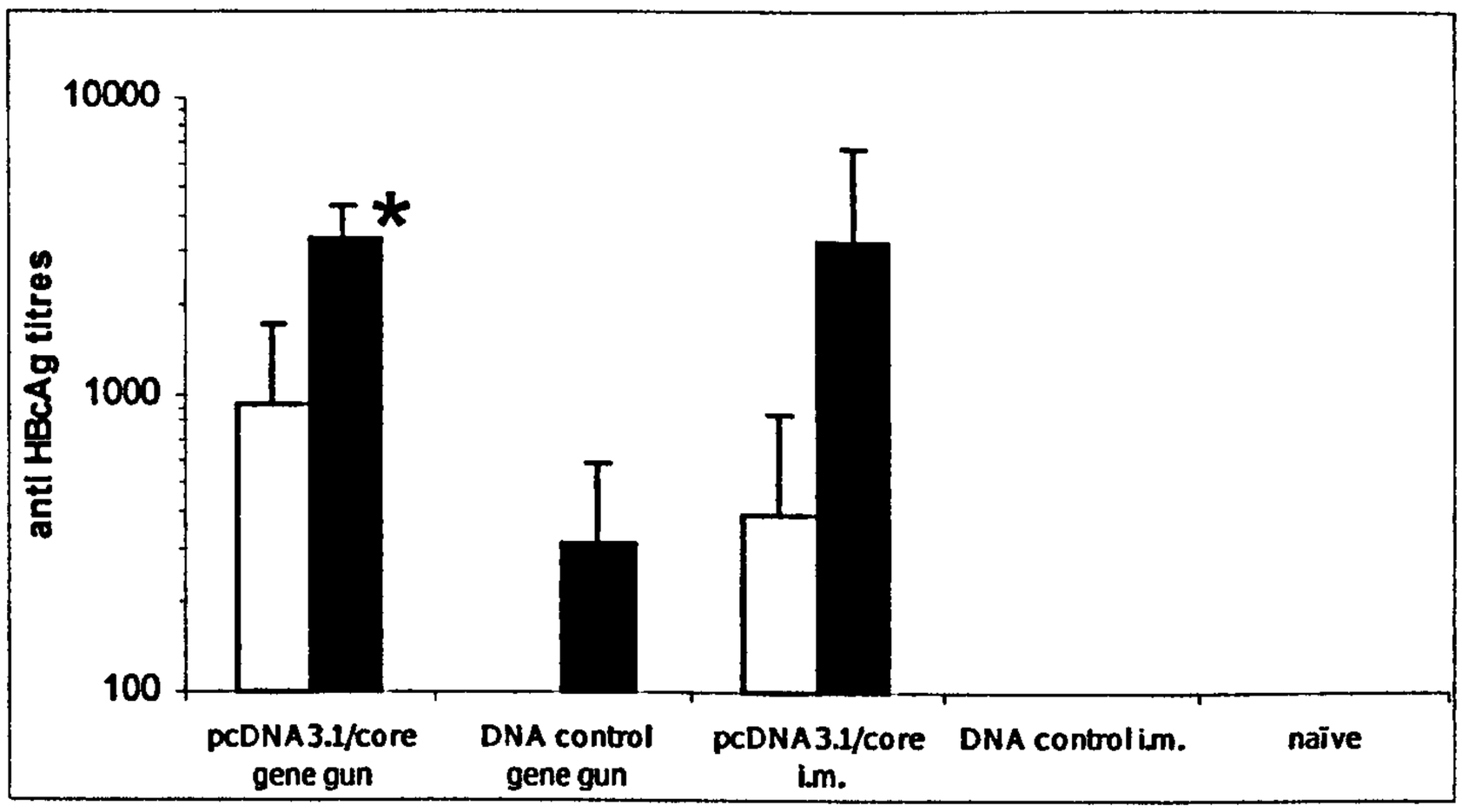


Figure 5-15: effect of boost on anti-HBcAg titres in gene gun and i.m. DNA vaccinated C57/Bl6 mice

Mice were immunised as described in Figure 5-8, with either 100μg DNA i.m. or 1μg DNA delivered by gene gun, on days 1 and 8. In addition, they were subsequently boosted with 1μg recombinant HBcAg protein i.m. on day 40 of the experiment, and the final sample taken on day 50. Sample bleeds were taken 1 day prior to the boost immunisation (□) or 10 days following the boost (■). Mean titres of groups of 5 mice (excepting pcDNA3.1/core gene gun group, which consisted of 10 mice) ± 1 standard deviation. * indicates a highly significant difference ($p>0.9999$) between titres in the gene gun group following the HBcAg boost.

5.9.2.2 CD4⁺ proliferation in Gene Gun Primed and HBcAg Boosted Mice

To compare the effect of gene gun priming on T cell responses, splenic $CD4^+$ cells from mice vaccinated with pcDNA3.1/core and boosted with protein. Again in contrast to the DNA alone experiments described at the start of this chapter, measurable proliferative responses were observed in cells from animals boosted with purified protein (Figure 5-17). The level of proliferation was considerably greater in the group primed with pcDNA3.1/core than that observed in either the naïve or vector alone control mice. Interestingly, although the highest specific responses were observed in inguinal lymph nodes from animals primed with pcDNA3.1/core (Figure 5-17B), the highest responses in the spleen were observed in animals immunised with the purified antigen alone. This may indicate a difference in the rate of trafficking of DNA- or protein-primed activated $CD4^+$ T cells to the spleen. As only one time point was considered in these experiments it is unclear whether the results reflect differences in timing or efficiency of trafficking to the spleens of these animals.

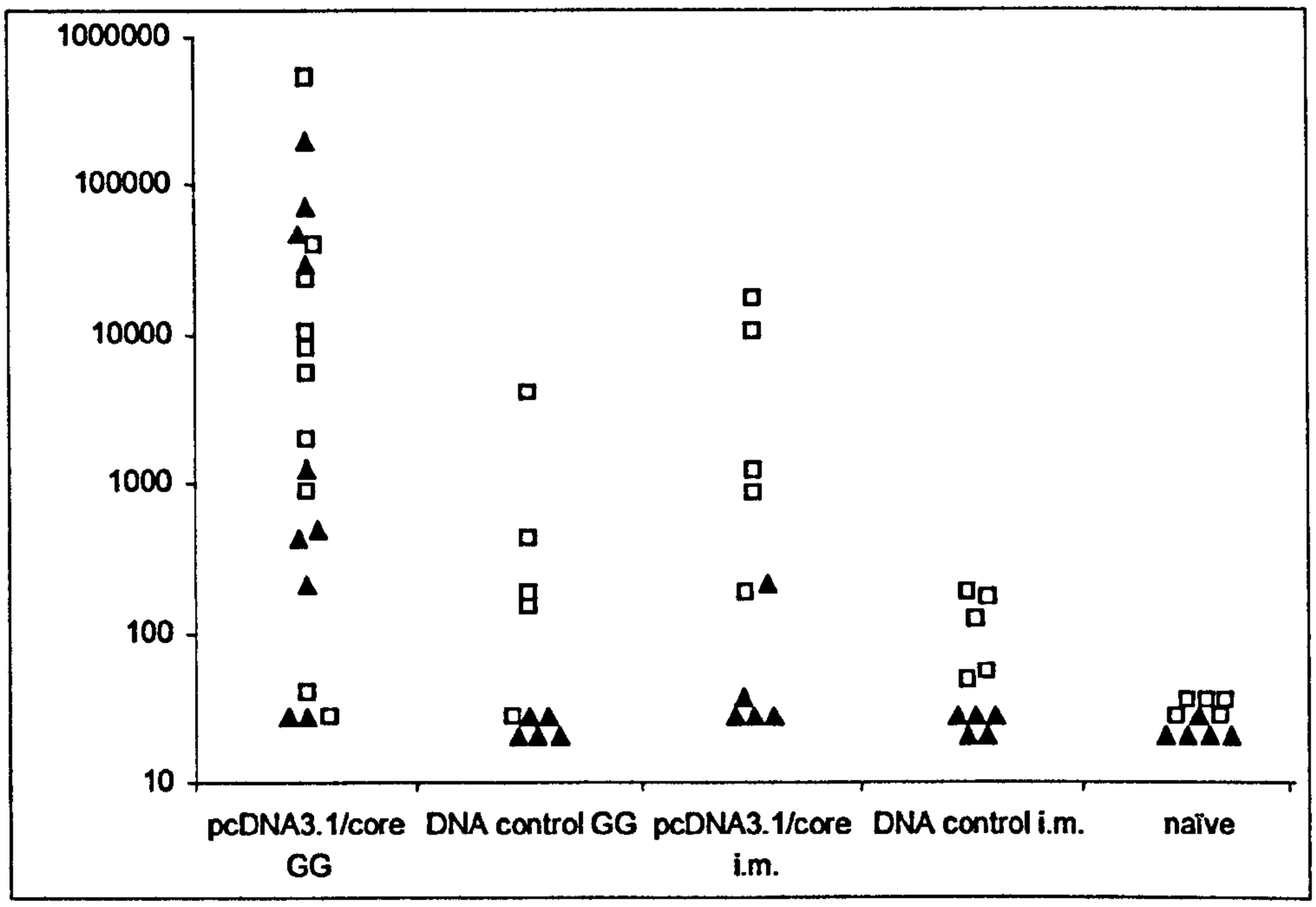
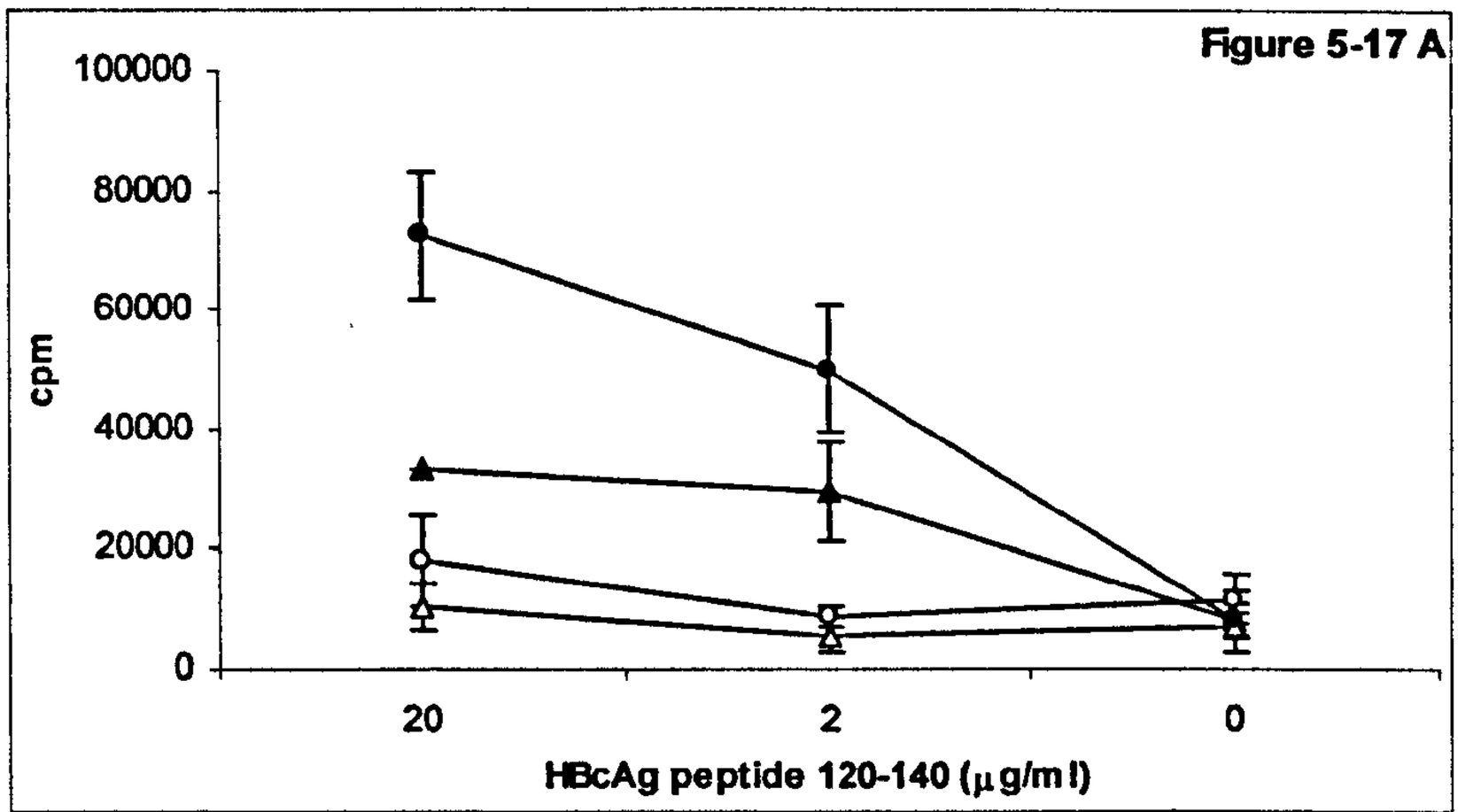


Figure 5-16: Antibody subtype titres following i.m. and gene gun vaccination.

C57/Bl6 mice were immunised with 100μg pcDNA3.1/core or control DNA on days 1 and 8, and boosted with 1μg HBcAg i.m. on day 40 (as described in Figure 5-8). Immunoglobulin subtypes were examined on day 50. Calculated specific anti-HBcAg titres are shown. ▲ represent IgG1 titres for individual mice, □ represent IgG2a titres.



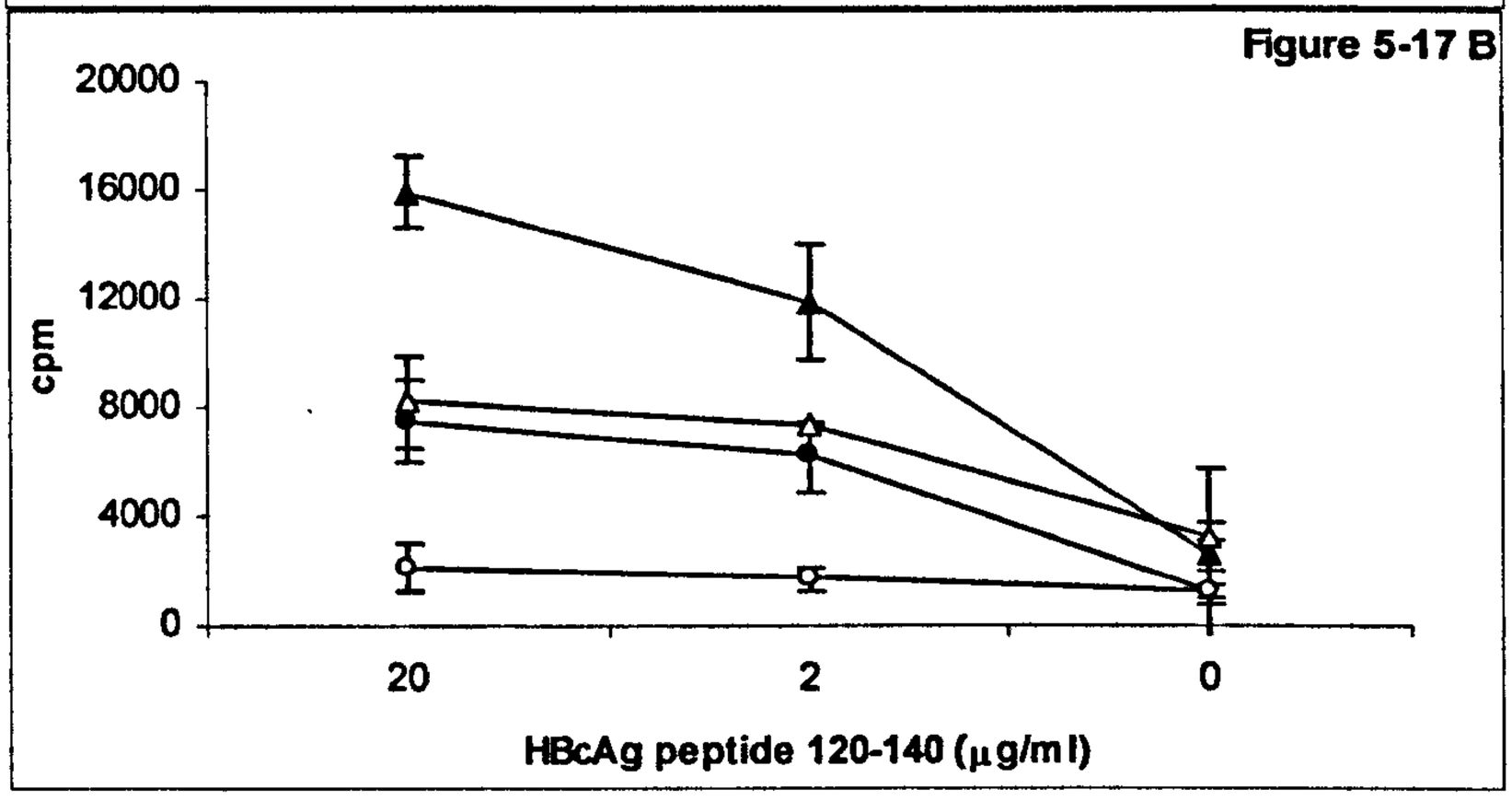


Figure 5-17: Gene gun DNA immunisation; spleen and lymph node $CD4^+$ T cell proliferation

C57/Bl6 mice were immunised on days 1 and 8 with 1-2µg pcDNA3.1/core (▲) or control DNA (Δ) by gene gun vaccination. One group was given 1µg purified HBcAg in alum i.m. on days 1 and 8 as a positive control (●). All three of these groups were given a boost of 1µg of purified HBcAg on day 34. One group remained untreated (o). Mice were sacrificed, splenocytes and draining lymph node cells recovered on day 45. The proliferation of spleen (Figure 5-17A) and lymph node (Figure 5-17B) cells from pooled mice was measured by β-emission from cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of peptide concentrations. Assay described in full in materials and methods sections 2.5.2 – 2.5.4. Mean CPM from triplicate cultures of pooled cells are shown ± 1SD. Note the difference in scale between spleen and lymph node graphs.

5.10 Summary of Responses to DNA Prime, Protein Boost Vaccination

5.10.1 Humoral Responses

The experiments described in this chapter show that pcDNA3.1/core is able to induce an antibody response in both C57 bl/6 and BALB/c mice, when delivered by i.m. or gene gun vaccination. This response can be significantly enhanced following boosting with the purified antigen. This shows that vaccinated animals have seen sufficient antigen as a result of the DNA vaccine to be specifically primed against this antigen. Responses to pcDNA3.1/core with an i.m. HBcAg boost appear equivalent and are unaffected by the route of delivery, despite 50 to 100 fold less plasmid delivered when the gene gun method is employed. These results show that both i.m. and gene gun DNA vaccination are effective at inducing HBcAg-specific humoral responses, especially when coupled with a purified protein boost.

However, despite the similarity in magnitude of the antibody response, i.m. DNA vaccination leads to an IgG2a dominated response, whilst gene gun immunisation results in a more mixed IgG1/IgG2a response. It has been shown that the structural nature of HBcAg is crucial to the nature of the T helper response developed: mice injected with purified HBcAg developed IgG2a but no IgG1 anti-HBcAg antibodies. In contrast, mice immunised with HBeAg, (an HBV protein with near identical sequence but which does not polymerise into a particulate form) exhibited a response dominated by IgG1 (Milich *et al.* 1997). This suggests that Th cells with the same specificity can develop into different Th subsets depending on the structural nature of the antigen.

The work in this chapter echoes these findings, with particulate antigen stimulating IgG2a production. Immunisation i.m. with the plasmid also stimulates a IgG2a dominated response suggesting that sufficient levels of antigen are being produced to form a particle. In contrast, gene gun vaccination results in a mixed response including both subtypes. It is unclear from these data whether the route of immunisation or the structure of the protein generated by the transfected cells at each location is responsible for this difference. However, previous work conducted in this laboratory showing a similar pattern of results when comparing i.m. and gene gun delivered DNA vaccines expressing a soluble, non-particulate antigen (Tetanus toxin fragment C) (Zlei 2002), suggesting the route of immunisation has a greater role to play in this phenomenon.

5.10.2 Cellular Responses

In summary, these results show that i.m. DNA vaccination with pcDNA3.1/core is capable of eliciting $CD4^+$ and $CD8^+$ T cell responses. The strong T helper proliferative response demonstrates that many helper cells had been exposed to and responded to the HBcAg expressed by the DNA vaccine. These $CD4^+$ T helper cells are important in co-ordinating the immune response, increasing the efficacy of specific humoral and mechanisms as well as encouraging non-specific innate immune functions.

The vaccine has also been shown to induce CTL killing of target cells bearing HBcAg epitopes. This is of interest because conventional vaccination strategies fail to induce CTL responses to HBcAg. This is thought to be due to the difficulty of processing large, particulate antigen in the MHC class I antigen-processing pathway. Demonstration of the CTL response in mice immunised with

pcDNA3.1/core confirms that the antigen is being expressed within eukaryotic cells where it can readily gain access to the class 1 pathway (see Introduction section 1.4.1.1). Unfortunately time did not permit similar analysis of antigen specific $CD8^+$ cells following immunisation with the gene gun.

This chapter shows that the DNA construct pcDNA3.1/core effectively generates specific cellular and humoral responses against the HBcAg. This is true of delivery by either the i.m. route or using a gene gun, and is especially promising when the effect of the combined prime-boost strategy is considered. The success of the prime/boost vaccination strategy, together with the knowledge that weak responses can be effectively increased by a heterologous boost encouraged the investigation of other strategies through which HBcAg-specific responses could be boosted. These alternative strategies are described in chapters 6 and 7.

6 RESULTS – DNA PRIME VACCINATION AND MUCOSAL BOOSTING WITH ATTENUATED *SALMONELLA TYPHIMURIUM*

6.1 Chapter Aims

The aim of the work described in this chapter was to examine the specific humoral and cellular immune responses generated when mice primed systemically with DNA encoding HBcAg were boosted mucosally by administration of an attenuated strain of *S. typhimurium* expressing the same antigen.

6.2 Introduction

Mucosal vaccine delivery has many advantages including the fact that oral immunisation can induce immune responses at mucosal surfaces as well as systemically, thus providing protection at the site of initial entry into the body for many pathogens. A full description of mucosal vaccination is given in section 1.5.3.

S. typhimurium is much studied as a vehicle for delivering heterologous antigens in mucosal vaccination. It is relatively simple to manipulate genetically, resulting in the production of a range of deletion mutants, many of which have been demonstrated to show an appropriate level of attenuation and immunogenicity (see Introduction sections 1.5.3.2 and 1.5.3.4 and Bumann et al or Darji et al for review (Bumann *et al.* 2000; Darji *et al.* 2000)). The strain used in these experiments, *S. typhimurium* BRD509, is also described in the Introduction (section 1.5.3.3).

6.3 Responses to pcDNA3.1/core, *S. typhimurium* BRD509 pGA-1 Vaccination

Following the successful boosting of primed responses with systemically administered recombinant HBcAg, we wished to consider whether similar levels of enhancement could be achieved when different boost vaccination strategies were employed. One such approach made use of the attenuated *Salmonella* strain described above; *S. typhimurium* BRD509 pGA-1. In these studies animals were primed systemically as described previously and then the effects of a subsequent boost of *Salmonella* expressing the HBcAg were observed.

6.3.1 Preparation of Bacteria for Intragastric Immunisation.

To ensure that the primed animals were boosted with an appropriate number of viable bacteria, a trial run of the inoculum preparation was undertaken. Bacteria were grown for 12-16 hours in anaerobic conditions at 37°C. The bacteria were harvested by centrifugation and resuspended in LB broth. As it is impossible to estimate the number of viable bacteria prior to the inoculation of the animals, a curve was generated that made use of the correlation between optical density at 650nm (OD_{650}) of the culture and viability. This was achieved by preparing the bacteria at various values of OD and performing viable counts (see Material and Methods section 2.1.6). The resultant graph is shown in Figure 6-1 and was used in the preparation of the inoculum for boosting the primed animals.

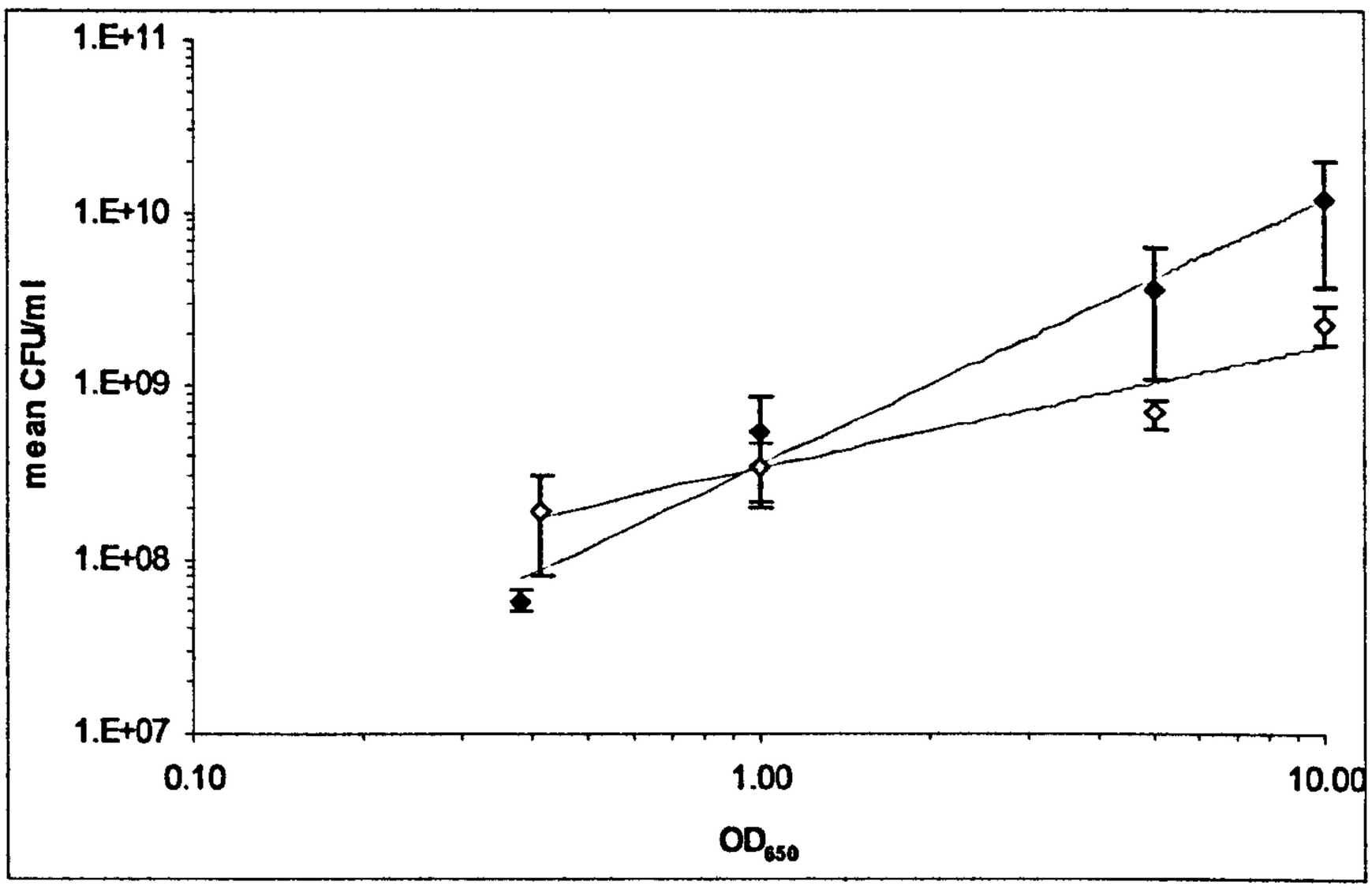


Figure 6-1: Standard curve to establish the relationship between viable numbers of *Salmonella* and optical density of cultures

The symbol ◆ represents the relationship between OD and viability of *S. typhimurium* BRD509 pGA-1, whilst the symbol ◇ represents *S. typhimurium* BRD509 alone. Values were calculated from colony counts from serial dilution samples cultured on agar as described in section 2.1.6.

6.3.2 Immunisation of Mice

Mice were primed i.m. with either 100μg of the HBcAg expression vector (pcDNA3.1/core) or the empty vector on days 1 and 8 of the experiment (as described previously). Sample bleeds taken at day 21 confirmed the previous observation that those animals immunised with the pcDNA3.1/core plasmid had generated specific anti-HBcAg antibodies. These were absent in mice immunised with the vector alone and naïve animals (Figure 6-2). Following DNA priming, the ten mice in each group (pcDNA3.1/core, pcDNA3.1 alone and naïve mice) were randomly divided into two groups of 5 animals. The first group were immunised intragastrically (i.g.) using a gavage needle with BRD509 pGA-1, whilst the second group were immunised with BRD509 alone (for vaccination schedule see Figure 6-3). The approximate number of viable bacteria administered in each case, is given in Table 6-1. This shows that despite efforts to correlate OD with viability, animals immunised with *Salmonella* alone were exposed to approximately 3 times the number of bacteria than those given *Salmonella* expressing the HBcAg. The impact of this on the results observed will be discussed later (see section 6.4). In addition, in an attempt to study the impact of the *Salmonella* immunisation alone, naïve animals were also inoculated with the *Salmonella* strain.

Mice were sacrificed 28 days after the i.g. boost and spleens and draining lymph nodes recovered. The extended time period between the boost and sacrifice (compared to the 10 day period for other experiments in this thesis) was intended to allow the bacteria to be cleared by the mice prior to assessment of immunity and thus prevent any bacterial infection of *in vitro* restimulation cultures.

	BRD509 $^{+}$ pGA-1	BRD 509 alone
Calculated number of bacteria per dose	2.5×10^{10}	8.1×10^{10}
Mean anti-LPS titre	1393	3003

Table 6-1: The relationship between bacteria administered and mean anti-LPS titre

The number of bacteria administered per dose is shown, calculated from cultures of serial dilutions made from the bacterial suspension administered to mice. Also shown is the calculated mean anti-LPS specific titre in the sera of animals 28 days after administration of bacteria.

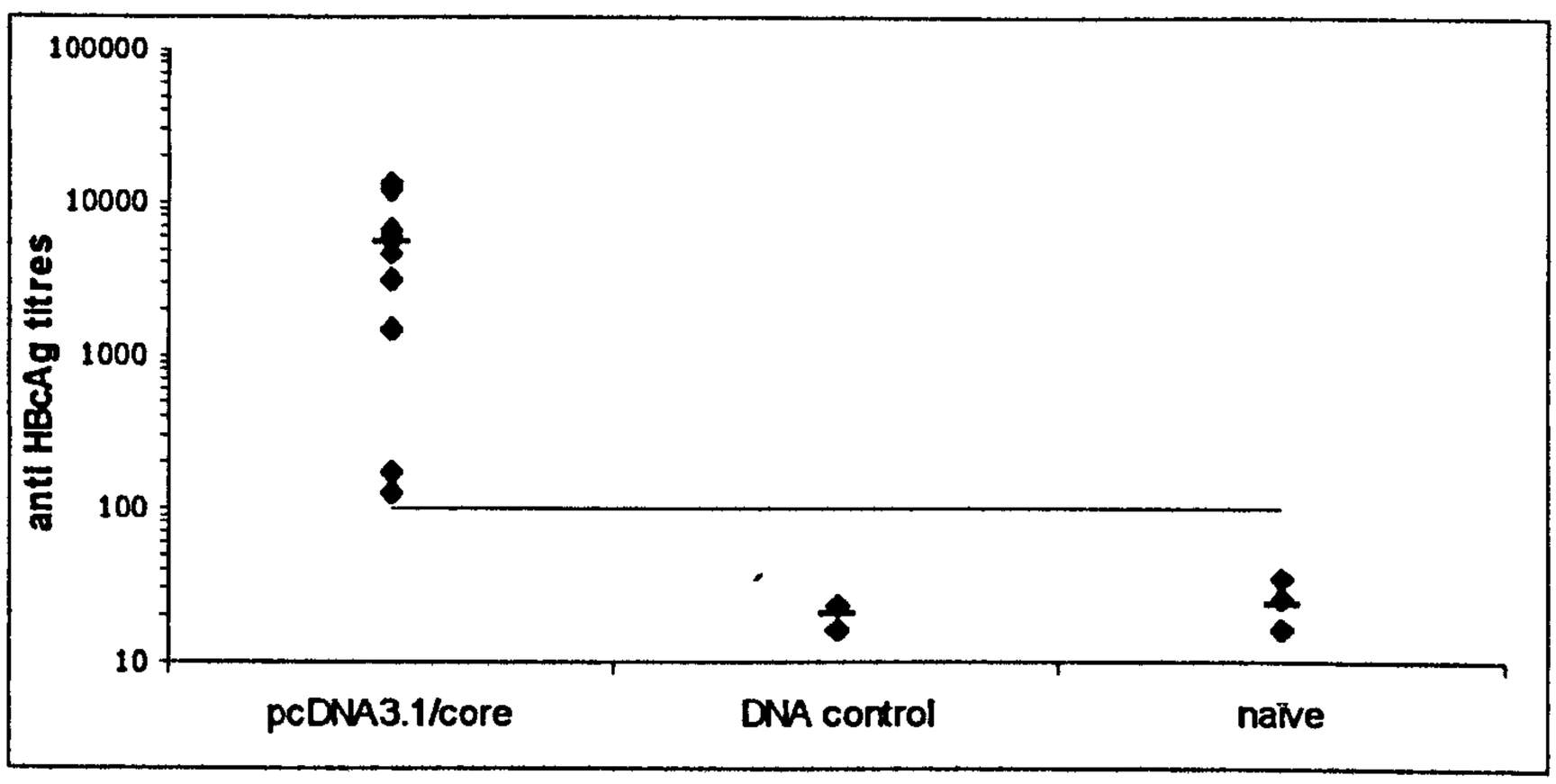


Figure 6-2: Titres of HBcAg-specific antibody in the sera of mice immunised with DNA alone

Titres of serum antibodies from individual mice (◆) were measured 14 days after the second of two 100μg doses of DNA given i.m. The mean calculated titre of each group of 5 mice is indicated as is the threshold of sensitivity of the assay (1:100, represented by horizontal line).

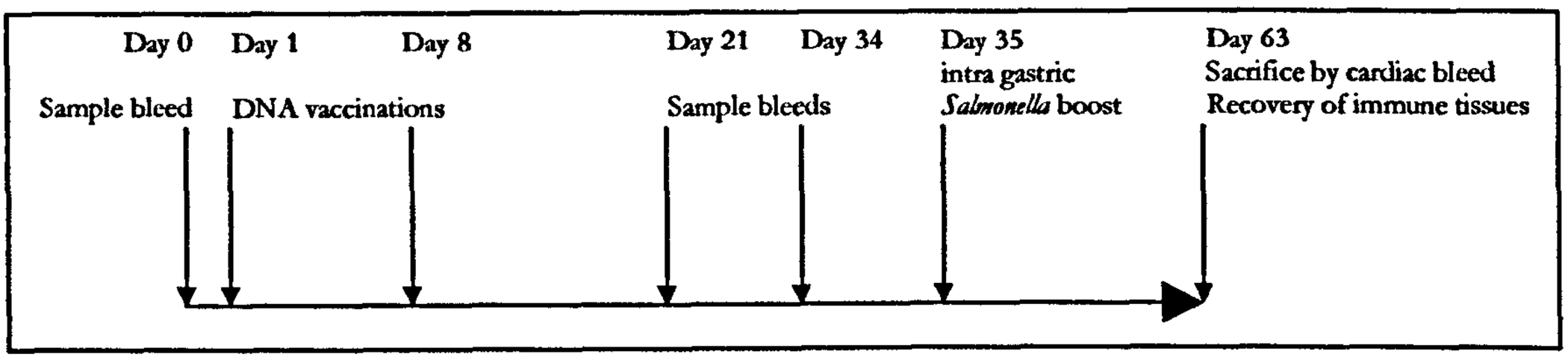


Figure 6-3: Vaccination regime

This figure shows the timing of vaccine delivery to the mice and of sample bleeding to assess the quantity of the anti-HBcAg generated by each mouse.

6.3.3 Humoral Responses to i.m. DNA Prime Vaccination Followed by i.g. Boosting with *Salmonella* Expressing HBcAg

Results in Figure 6-4 show the titres of antibodies in the sera of mice immunised i.m. with DNA and boosted with *Salmonella*. These data indicate that animals primed with pcDNA3.1/core and boosted with BRD509 pGA-1 show a mean antibody response more than 10-fold higher than those primed with pcDNA3.1/core and boosted with the control *Salmonella*. However, due to the high variability of individual titres within this group the results are not statistically significant.

Surprisingly, in contrast to the literature, no immune response was detected in animals given *Salmonella* expressing the HBcAg without DNA priming. However, these responses were measured following a single dose of *Salmonella* and may well have increased over time or following administration of a second dose of the bacteria (as was used in the described paper) (Londono *et al.* 1996).

Of the un-primed mice and mice primed with control DNA, only the group primed with control DNA exhibited a specific anti-HBcAg antibody response following immunisation with the HBcAg-expressing bacteria. The mean titre in this group was approximately half that of mice immunised with pcDNA3.1/core and boosted with control *Salmonella*. Since no response was observed in un-primed mice, it appears that once again, the DNA alone is responsible for some non-specific immunostimulatory effects. This is especially impressive given the length of time between the second DNA vaccination and the *Salmonella* inoculation, and the physical separation of the sites of administration (i.e. peripherally with the i.m. vaccination versus the mucosal boost).

6.3.4 Humoral Responses in Gene Gun-Primed Mice

In contrast to the result observed in the i.m. primed animals, boosting of animals with *Salmonella* in mice primed with DNA using the gene gun failed to enhance the antibody response generated, with no significant difference between the titres before and after the boost (Figure 6-5). The lack of synergy between the two vaccinations may be due to the different quality of the immune responses generated. As noted previously (see Chapter 5), the gene gun vaccination elicits a more Th2-like immune response, whereas i.m. vaccination gives an IgG2a only, Th1 response. Oral *Salmonella* immunisation has been described as stimulating a Th1 response similar to that achieved by priming with DNA i.m. (Dunstan *et al.* 1998) In this case, production of pro-Th1 cytokines by Th1 cells activated during the boost might enhance the activity of antigen specific Th1 cells generated in response to the priming vaccination. This mechanism may be less successful in gene gun vaccinated animals, since Th2 cells from the DNA prime would not respond to cytokines produced by Th1 cells activated by the *Salmonella*.

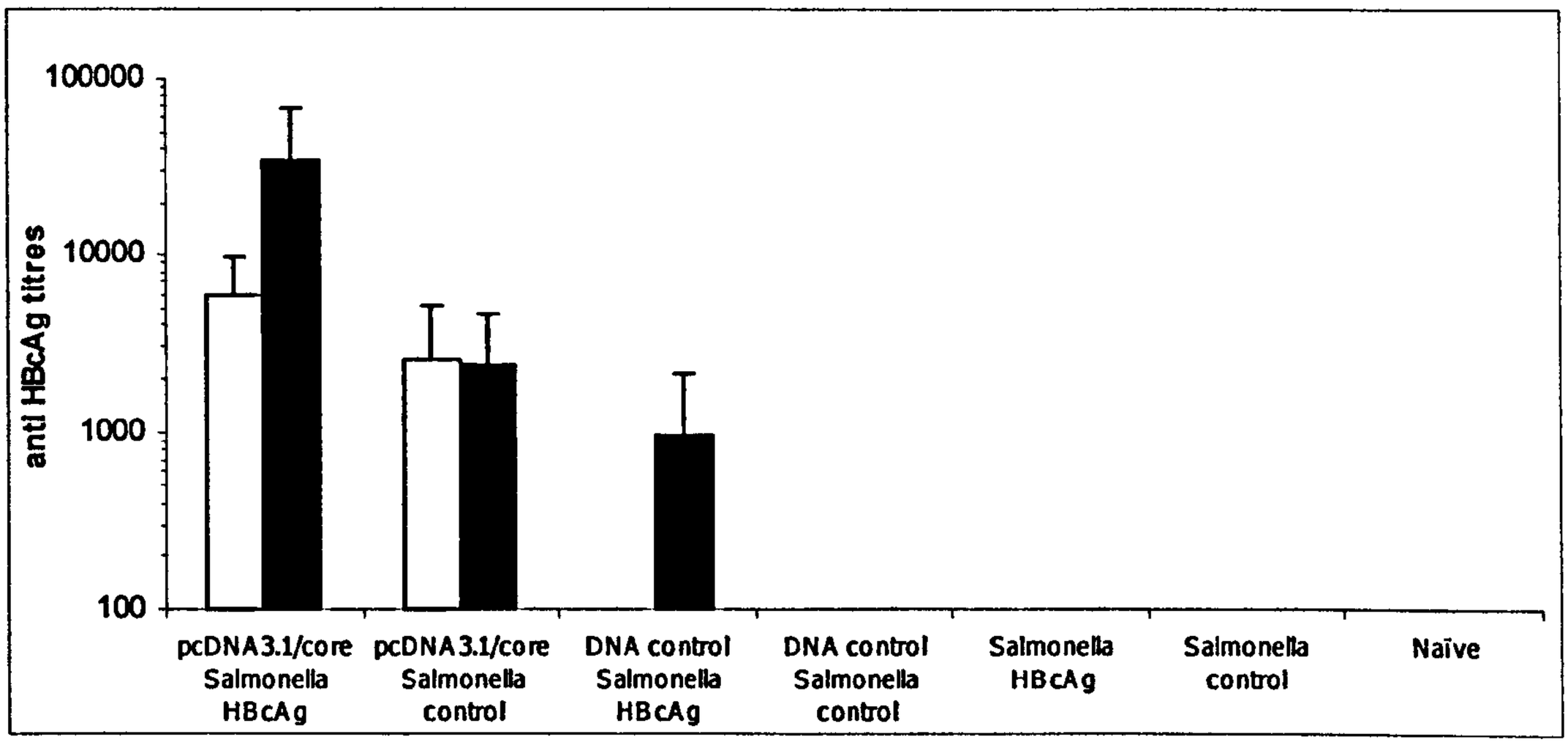


Figure 6-4: Titres of HBcAg-specific antibody in the sera of C57/Bl6 mice immunised with DNA i.m. and boosted with *Salmonella*

Groups of 5 C57/Bl6 mice were immunised i.m. with 100μg of either pcDNA3.1/core or control DNA. This was repeated on day 8 of the experiment. Mice were boosted with 10^{10} *S. typhimurium* BRD 509 with or without the pGA-1 plasmid (*Salmonella* HBcAg and *Salmonella* control) on day 35. Mean titres at day 34 (prior to boost; □) and day 63 (post boost, ■) are shown. Error bars represent 1 standard deviation.

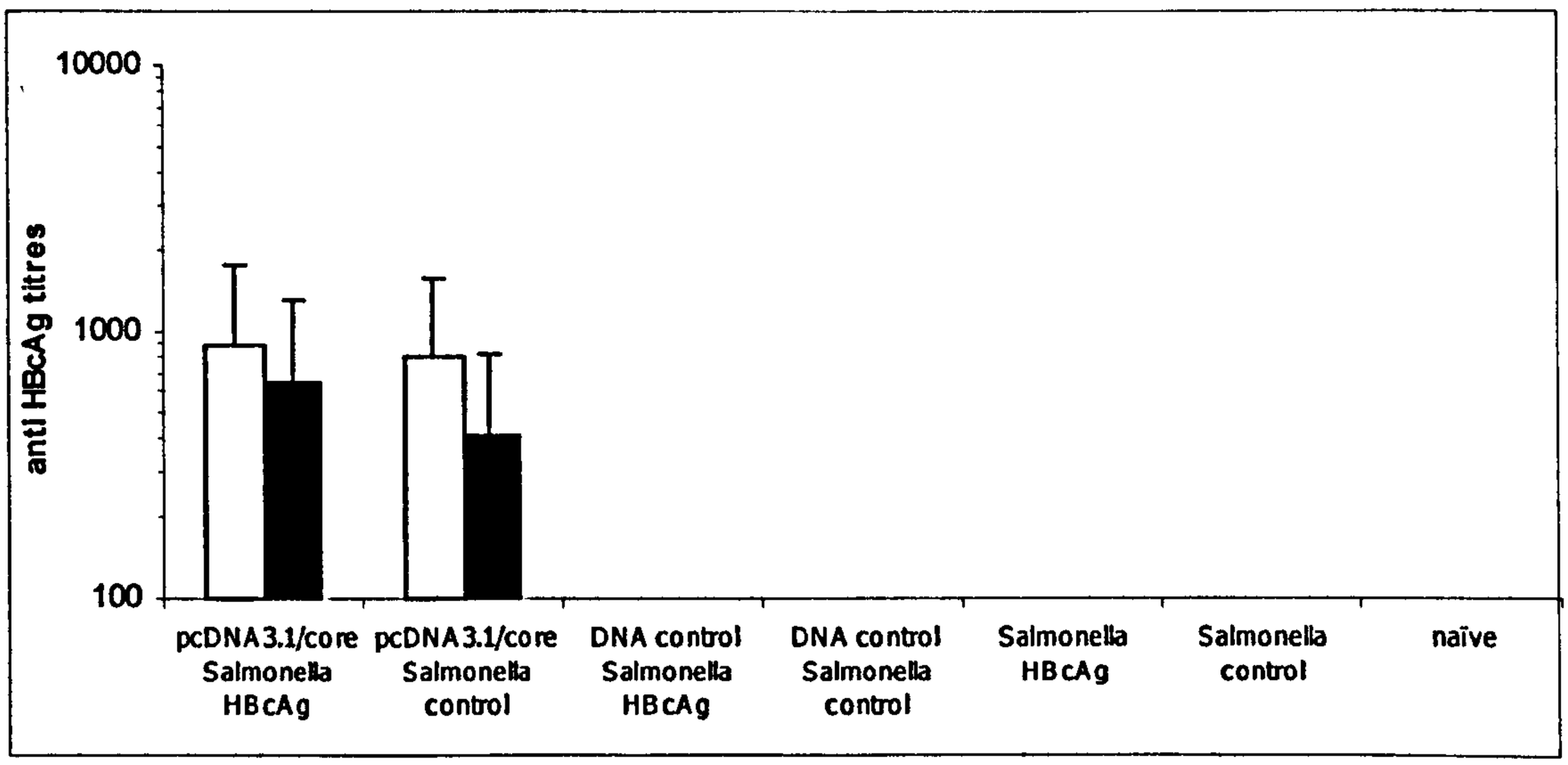


Figure 6-5: Titres of HBcAg-specific antibody in the sera of C57/Bl6 mice immunised with DNA by gene gun and boosted with *Salmonella*

Groups of 5 C57/Bl6 mice were immunised by gene gun with 1-2μg of either pcDNA3.1/core or control DNA. This was repeated on day 8 of the experiment. Mice were boosted with 10^{10} *S. typhimurium* BRD 509 with or without the pGA-1 plasmid on day 35. Mean titres at day 34 (prior to boost; □) and day 63 (post boost, ■) are shown. Error bars represent 1 standard deviation from the mean titre calculated from 5 mice.

6.3.5 Generation of Mucosal IgA Antibody

An obvious advantage of combining a systemic and mucosal vaccination strategy would be the induction of mucosal as well as systemic immune responses. To determine whether the prime boost strategies used above could stimulate the production of HBcAg specific mucosal IgA, ELISAs were performed on faecal samples and intestinal washes taken from the mice of both gene gun and i.m. prime experiments on day 63. Unfortunately, none of the samples showed the presence of any HBcAg specific IgA. This was disappointing; especially in the groups given *Salmonella* expressing the antigen alone, which according to the literature should have generated these detectable responses. The implication of this poor response will be discussed in greater detail later in the chapter.

6.3.6 Anti-*Salmonella* Responses

6.3.6.1 Anti-Salmonella Serum Antibodies

Serum samples taken at the end of the experiment were examined for the presence of anti-*S. typhimurium* LPS antibodies by ELISA (described in section 2.5.1.3). The results of this assay (shown in Figure 6-6) show that the mice immunised with either strain of *Salmonella* develop a low but specific anti-LPS response. In addition there is a good correlation between the calculated bacterial dose and the mean anti-LPS titre with animals immunised, with 3 times more *Salmonella* showing approximately 3 times higher titres of antibodies to LPS in the sera (see Table 6-1). These titres indicate that the BRD509 strain is immunogenic with or without the plasmid pGA-1, and that the bacteria persist for sufficient time for a serum antibody response to develop.

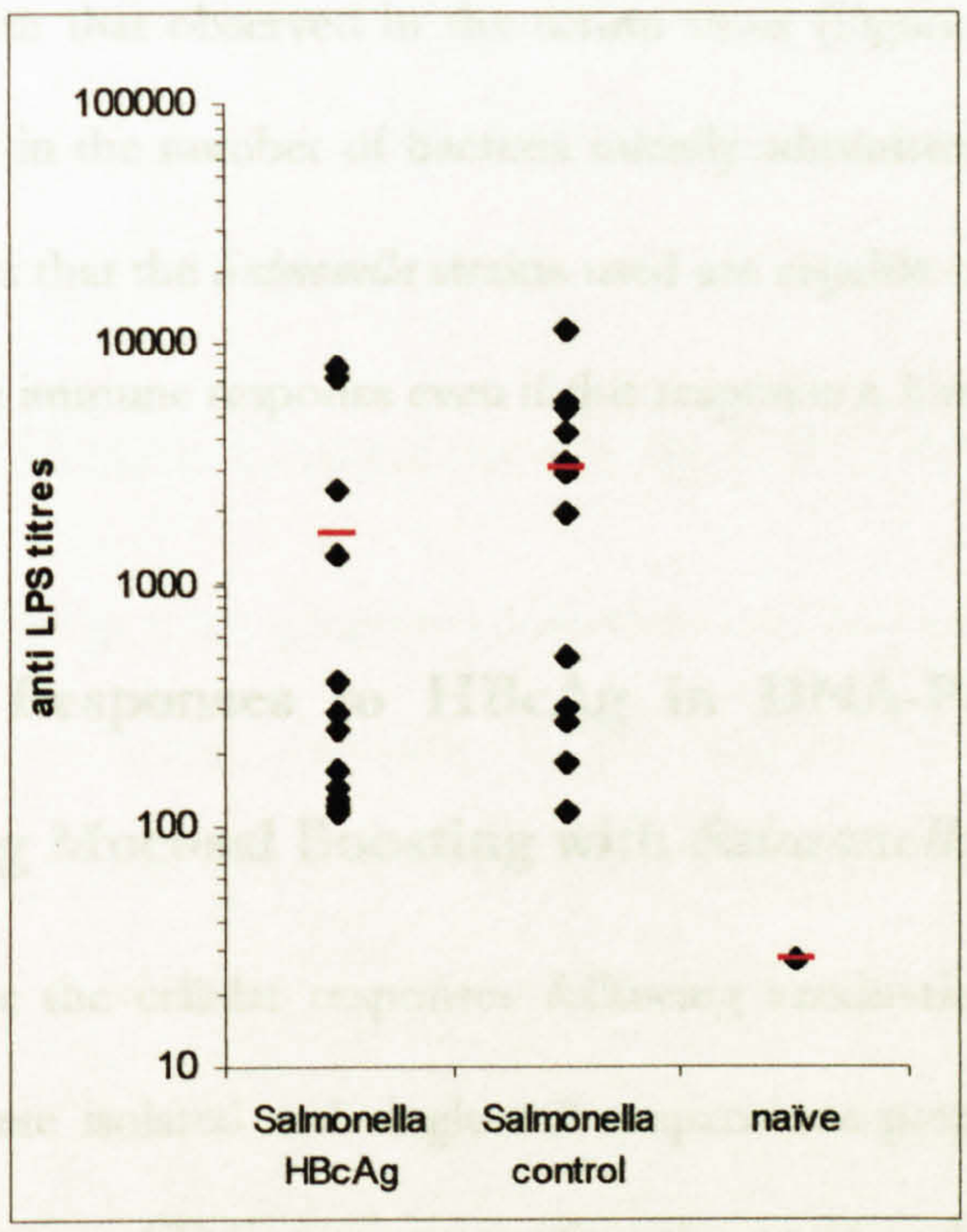


Figure 6-6: Anti-*S. typhimurium* LPS titres in serum 28 days after administration

Mice were immunised with *S. typhimurium* BRD509 pGA-1 (n=13), *S. typhimurium* BRD509 alone (n=12), or remained naïve (n=5). The calculated inoculation doses are shown in Table 6-1. Titres of LPS-specific antibodies in the sera of individual mice (◆) and calculated mean titres (horizontal bars) are shown.

6.3.6.2 Anti Salmonella Mucosal Antibodies

Figure 6-7 shows the anti *S. typhimurium* LPS titres of individual mice measured from intestinal washes (IW) and faecal pellets (FP). In the intestinal wash samples, the majority of mice from both groups (those immunised with BRD509 pGA-1 and those with the control bacteria) display LPS specific IgA responses. These responses are about three times stronger in the control group (*Salmonella* alone) than the group immunised with *Salmonella* expressing the HBcAg. This result is in agreement with that observed in the serum titres (Figure 6-6) and probably reflects differences in the number of bacteria initially administered (see Table 6-1). This result indicates that the *Salmonella* strains used are capable of generating both a mucosal and serum immune response even if this response is limited the carrier.

6.3.7 Cellular Responses to HBcAg in DNA-Primed Animals Following Mucosal Boosting with *Salmonella*.

To measure the cellular responses following vaccination, tissues from all groups of mice were isolated and single cell suspensions prepared. These were either used to measure $CD4^+$ responses using the optimised method described (Materials and Methods section 2.5.5) or cultured with RBL5 target cells for the $CD8^+$ IFN-γ ELISPOT assay (Materials and Methods section 2.5.7). Unfortunately, despite the presence of antibiotics in the tissue culture medium in both assays, contamination was observed in the cultures of cells taken from mice immunised with the *Salmonella* expressing the HBcAg protein. This contamination prevented any measurement of the cellular responses. Interestingly, bacterial growth was restricted to cultures taken from those mice immunised with BRD509 pGA-1. In order to determine whether the contaminants were the *Salmonella* used for

vaccination persisting in the tissues, an HBcAg-sequence specific PCR was conducted. This confirmed the presence of the HBcAg gene in the contaminating bacteria, suggesting that the contamination due to the presence of the vaccine strain, which was able to persist *in vivo* for at least 28 days following immunisation.

Although many experiments using *Salmonella* as a delivery system have been performed successfully at 28 days following vaccination, the study which most closely resembles this one (Londono *et al.* 1996) studied cellular responses 42 days after vaccination. It is likely that by this time point the bacteria would be cleared from the organs, allowing measurement of cellular responses.

6.4 Study of Tissue Colonisation by BRD509

The anti-HBcAg titres generated following i.m. and gene gun vaccination were generally disappointing, especially as this approach has been apparently successful previously (Londono *et al.* 1996). However, on further consideration of the literature, it became clear that most of the studies described used BALB/c (H2-d) (Schodel *et al.* 1994); (Huang *et al.* 2001) rather than C57/Bl6 mice (H2-b). Although *aro* mutants of *S. typhimurium* have been observed to be attenuated in this strain of mice (Khan *et al.* 2003), it has also been shown that inbred mouse strains display varying levels of natural resistance to *Salmonella* infection (Hormaeche 1979). It was therefore unclear whether the lack of response reflected differences in the ability of the two strains of mice to clear the bacteria.

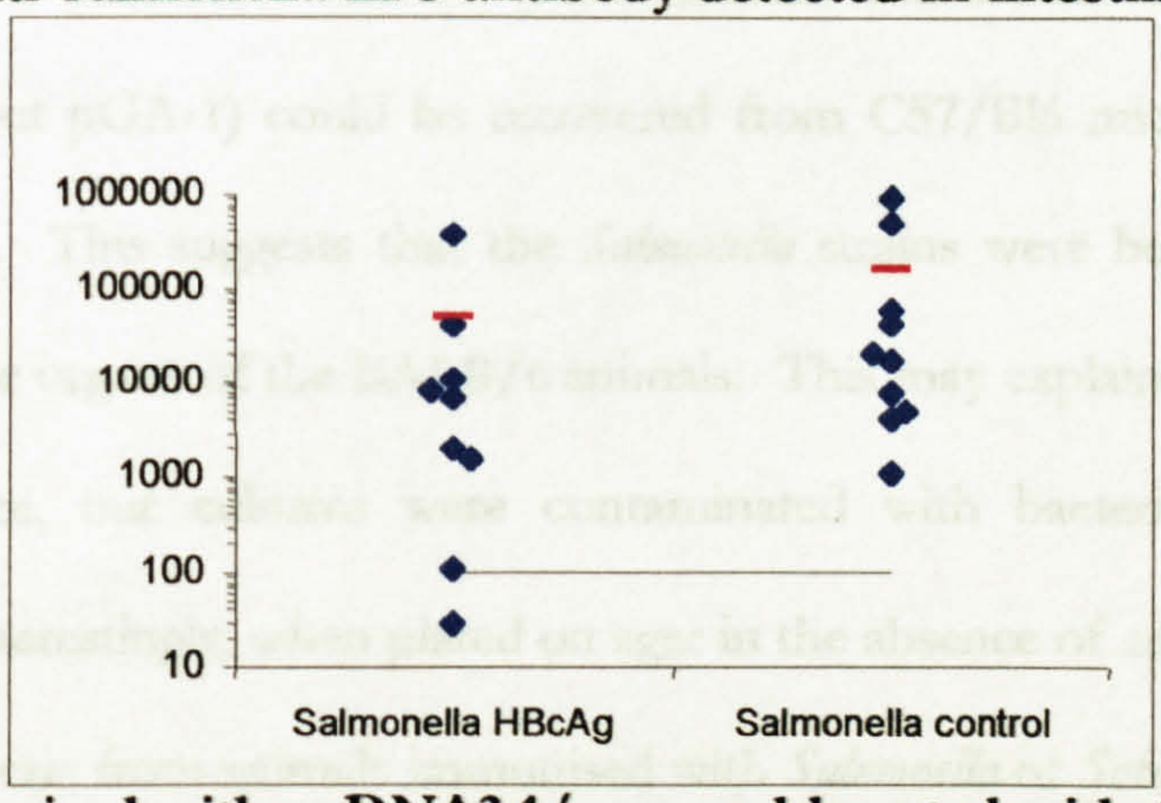


Figure 6-7: Anti-*Salmonella* LPS antibody detected in intestinal washes from bacteria immunised with pcDNA3.1/core and boosted with *Salmonella* with or without the pGA-1 plasmid.

Specific antibody titres from individual mice (◆) were determined as the reciprocal of the highest serum dilution which gave a value of 0.4 above the background. The mean calculated titre of groups of mice is indicated as is the threshold of sensitivity of the assay (1:100 – represented by the horizontal line).

In an attempt to determine whether colonisation of organs varied in BALB/c and C57/Bl6 mice, parallel experiments were performed in which mice of both haplotypes were immunised with the two *Salmonella* strains used in this experiment. Twenty four days after immunisation, animals were culled and Peyer's patches, mesenteric lymph nodes, livers and spleens were removed. These tissues were homogenised and viable bacterial counts prepared on L-agar plates with and without ampicillin. Results of the experiment are shown in Figures 6-8 and 6-9.

Figure 6-8 shows the number of viable bacteria (CFU/tissue) recovered from various tissues and grown on L-agar plates containing no antibiotics. These data clearly show that more bacteria (from animals immunised with bacteria both with and without pGA-1) could be recovered from C57/Bl6 mice than from the BALB/c strain. This suggests that the *Salmonella* strains were being more readily cleared from the organs of the BALB/c animals. This may explain why, in contrast to the literature, our cultures were contaminated with bacteria 28 days post vaccination. Interestingly, when plated on agar in the absence of antibiotic, the total number of bacteria from animals immunised with *Salmonella* or *Salmonella* containing the plasmid did not appear to vary significantly within each mouse strain. This suggests that the presence of the plasmid does not result in any additional metabolic burden on the strain. However, if the number of viable, ampicillin-resistant bacteria recovered from the same animals are calculated (Figure 6-9), it is clear that the presence of the plasmid has some effect, as a high percentage of the total bacteria have managed to effectively lose this construct. This means the number of bacteria capable of re-stimulating the DNA primed response to HBcAg will be fairly low. This lack of stability is surprising, given the previous use of the plasmid in *Salmonella* inoculation studies that resulted in the development of specific anti-HBcAg titres (Londono *et al.* 1996). The explanation could be expression of the antigen occurs

over an initial period shortly after administration of the bacteria, and although the plasmid was lost at some stage, expression up to that point was sufficient to enhance the existing systemic antibody response to HBcAg.

Interestingly, the majority of bacteria were recovered from the Peyer's patches and mesenteric lymph nodes in both strains of mice. This result probably reflects the relatively low numbers of bacteria that survive and are able to traffic to deeper tissues, compared to much larger numbers able to colonise the epithelium and M cells of the Peyer's patches. The high counts in the mesenterics may indicate the presence of macrophages recently infected via the nearby GALT but which have not yet migrated to other sites.

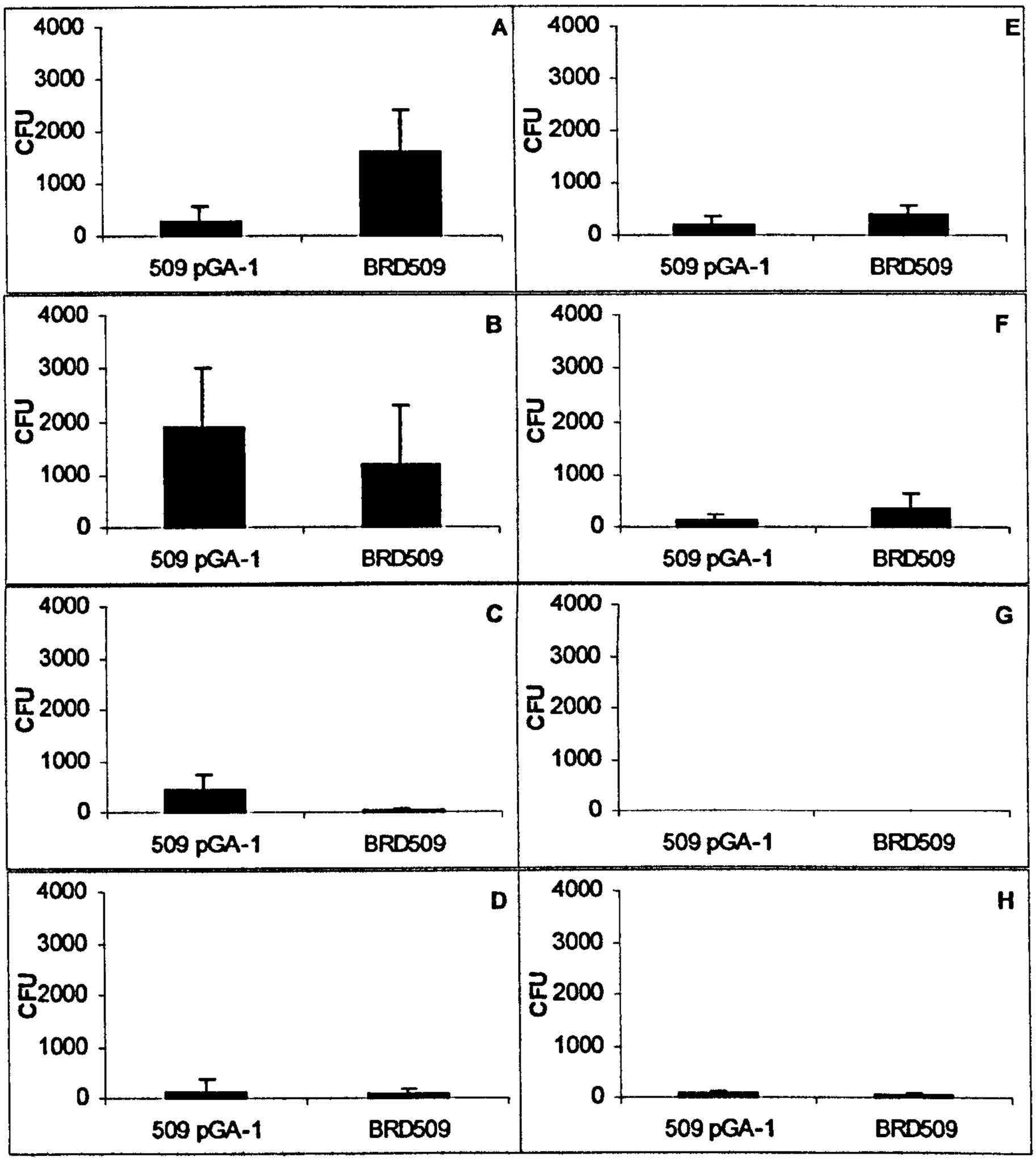


Figure 6-8: Number of viable *Salmonella* recovered from various tissues of BALB/c and C57/Bl6 mice 24 days after i.g. vaccination

BALB/c and C57/Bl6 mice were immunised i.g. with $2.3x10^{10}$ *S. typhimurium* BRD 509 with or without the pGA-1 plasmid. Mice were sacrificed and Peyer's patches, mesenteric lymph nodes (MLN), spleens and livers were recovered. Tissues were homogenised and serial dilutions made in PBS. Dilutions were plated on LB agar and incubated overnight at 37°C. CFU per organ were calculated from colony counts. Figures 6-8A – D show the number of bacteria recovered from C57/Bl6 mice whilst Figures 6-8E – H show the number recovered from BALB/c mice. A and E are counts from the Peyer's patches, B and F from the mesenteric lymph nodes, C and G from the spleen and D and H from the liver.

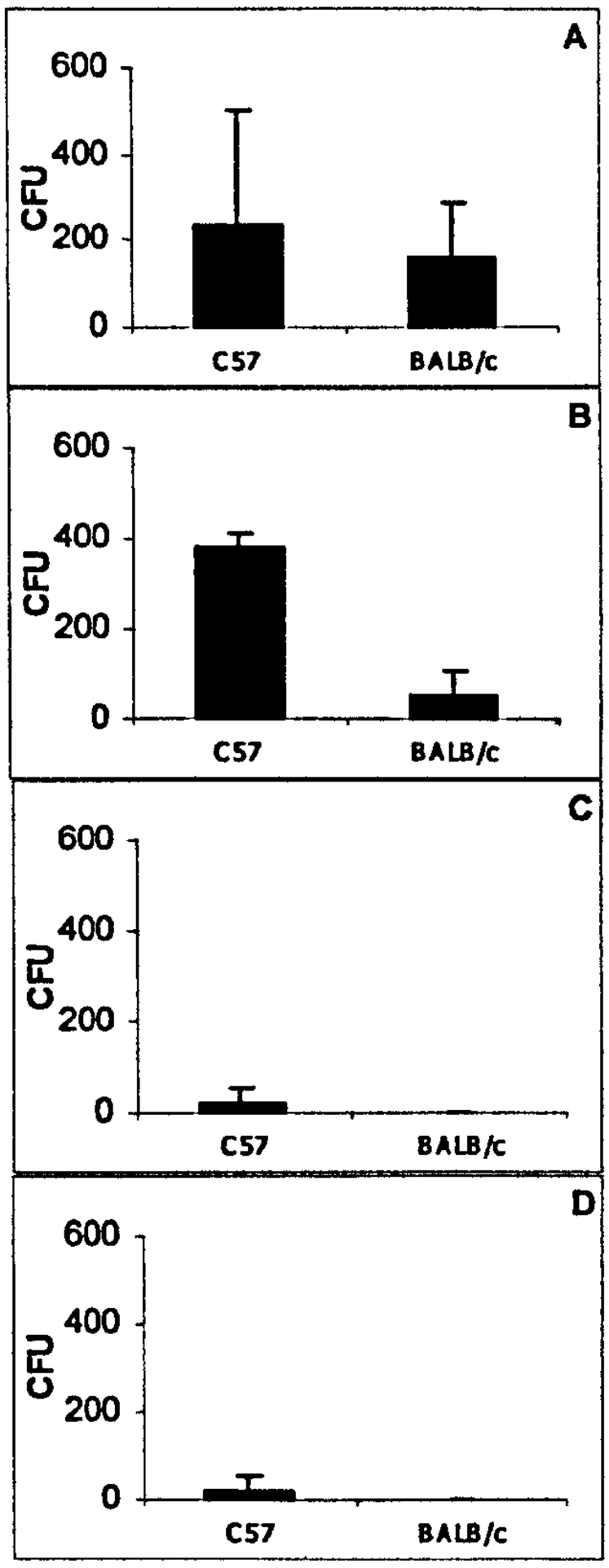


Figure 6-9: Number of viable, ampicillin resistant bacteria recovered form the tissues of BALB/c and C57/Bl6 mice 24 days after i.g. vaccination

Samples from the same mice as described in Figure 6-8 were cultured on LB plates containing ampicillin. This allows growth of only *S. typhimurium* BRD509 carrying the pGA-1 plasmid. A shows counts from the Peyer's patches, B from the mesenteric lymph nodes, C from the spleen and D from the liver.

6.5 Summary of Results

In these experiments, priming of a humoral response was only boosted by i.g. vaccination with *S. typhimurium* BRD509 pGA-1 when DNA was delivered i.m. Such an enhancement was not observed when the animals were primed intradermally using the gene gun (Figure 6-4).

Obviously the efficacy of the prime-boost strategies described in this chapter cannot be fully assessed without consideration of the T helper cell and CTL activation. This could be addressed by extending the period between *Salmonella* vaccination and harvesting cells for T cell assays to 42 days (Londono *et al.* 1996). Alternatively, different antibiotics could be used in the *in vitro* cultures that would more effectively prevent bacterial growth. $CD4^+$ T cell proliferation data would give a much clearer understanding of the role of the mechanisms driving the anti-HBcAg antibody response, as well as clarifying the presence and magnitude of a $CD8^+$ T cell response.

7 RESULTS – SYSTEMIC DNA AND INTRANASAL PROTEIN VACCINATION

7.1 Chapter aims

The aim of the work described in this chapter was to determine the impact of mucosal boosting with purified protein on the magnitude and type of immune response generated in mice that had been previously vaccinated systemically with pcDNA3.1/core.

7.2 Introduction

Work described in Chapter 5 examined the effect of systemic boosting, using purified protein, on the immune response generated following systemic priming of the animals with the DNA vaccine pcDNA3.1/core. In this chapter, experiments were designed to determine to what extent mucosal boosting using purified protein could affect the nature and magnitude of the primed responses generated.

These experiments aimed to answer the following questions:

1. Is the HBcAg particle an effective mucosal antigen, or does it require the presence of a mucosal adjuvant to trigger the mucosal response? For a description of the mucosal adjuvants used in this project, see sections 1.3.1 and 1.5.4.3.
2. Does priming with systemic DNA vaccination prior to mucosal boosting with the particulate HBcAg enhance the subsequent immune

response? If so, what effect does the route (that is, i.m. or i.d.) of the priming vaccination have on the final immunological readout?

7.3 Efficacy of HBcAg as a Mucosal Immunogen

7.3.1 Humoral Responses to i.n. Delivered Purified HBcAg

In initial experiments mice were immunised intranasally with purified HBcAg in the presence or absence of the mucosal adjuvant LT. In addition, as a positive control, a third group of animals were immunised i.m. with 1μg of HBcAg, without adjuvant. Ten days later the HBcAg specific serum antibody response was measured by ELISA. The data shown in Figure 7-1 indicates that even after this relatively short period of time, mice that received HBcAg i.n. with LT developed a low but detectable anti-HBcAg response in the sera. These responses were of a similar order of magnitude to those observed when the same amount of protein was delivered parenterally without adjuvant. In contrast, mice immunised with the same dose of HBcAg i.n. in the absence of an adjuvant, only a very low antigen specific response was observed. Therefore the immunogenicity of the protein is dependent on the route of delivery. Nose and lung washes were also examined for the presence of anti-HBcAg antibody, but no specific responses were observed in any of the samples tested.

7.3.2 $CD4^+$ T Cell Proliferation

In addition to humoral responses, proliferation of HBcAg specific $CD4^+$ T was also measured in these mice. For these experiments cells were isolated from the

spleens and cervical lymph nodes (CLN) and restimulated *in vitro* with HBcAg specific peptide (Materials and Methods section 2.5.2 – 2.5.5).

The results of these experiments are shown in Figure 7-2. Following i.n. delivery of HBcAg with LT, strong proliferation of antigen specific $CD4^+$ T cells can be observed in both the spleens and CLN. Interestingly, the proliferation appears greater in the local draining lymph nodes than the spleen, with the average count at the highest concentration of restimulating antigen more than 20,000 higher than that in the spleen. In contrast, when the antigen was delivered without LT, there was no detectable response in the lymph nodes and only a moderate response in the spleen. This demonstrated that the presence of the LT adjuvant is very effective at stimulating a long-lasting antigen specific $CD4^+$ response in the local tissues.

The $CD4^+$ proliferation in response to HBcAg delivered i.m. also appears to be different from the situation with LT as in this case, like the antigen alone mucosally, low responses are seen in spleens but not in the local draining lymph nodes (popliteal and inguinal), as shown in Figure 5-5, for example. This demonstrates that the localisation and magnitude of immune responses may be dependent on both the route of immunisation and the adjuvant used to enhance this response.

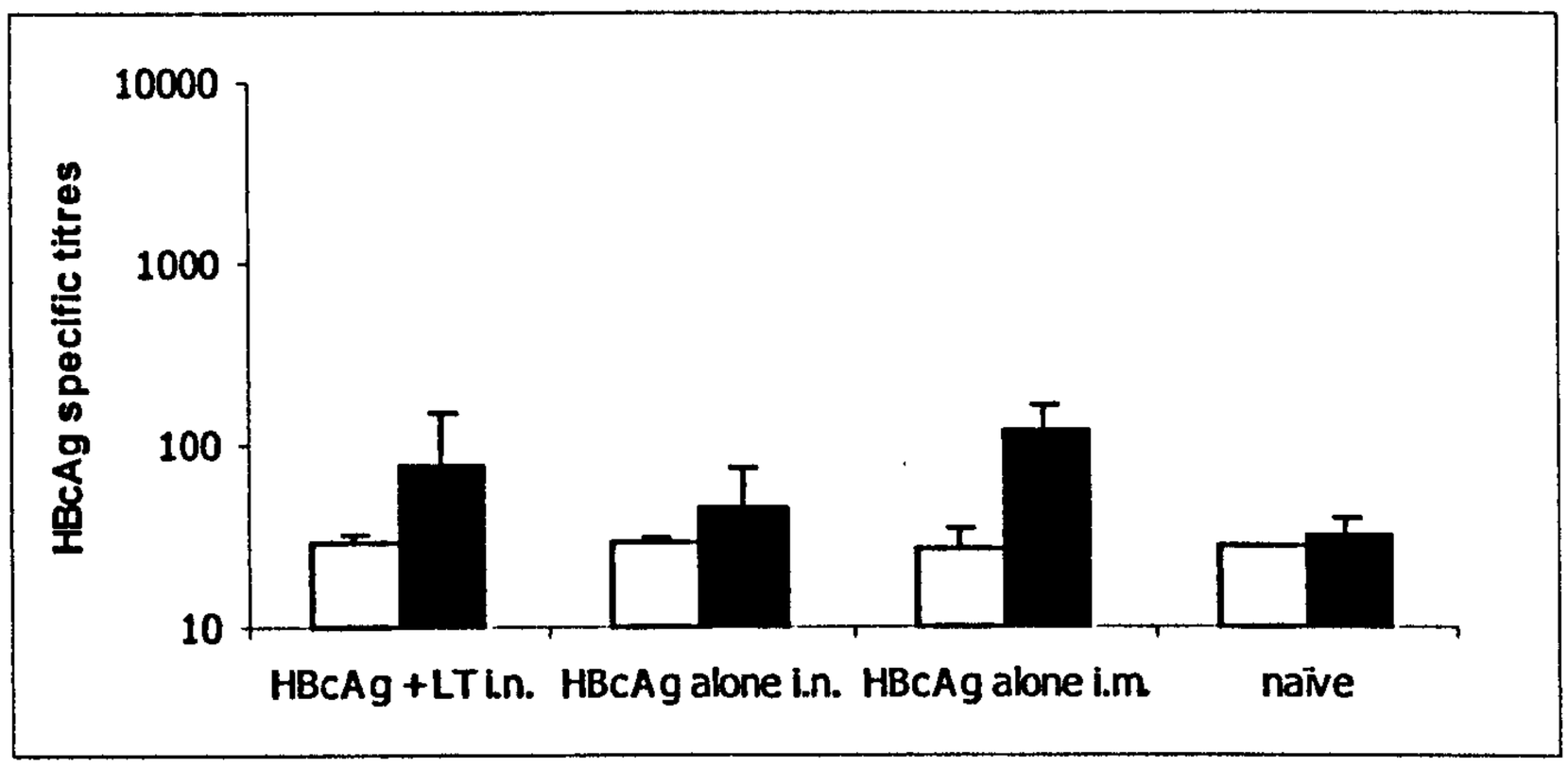


Figure 7-1 Serum anti-HBcAg titres following i.n. or i.m. HBcAg vaccination in C57/Bl6 mice

Groups of 10 C57/Bl6 mice were immunised i.n. with 1μg HBcAg with or without LT, or with 1μg of HBcAg i.m. The mean serum anti-HBcAg titres are shown prior to immunisation (□) or 10 days post immunisation (■), ± 1S.D. Results are representative of two experiments.

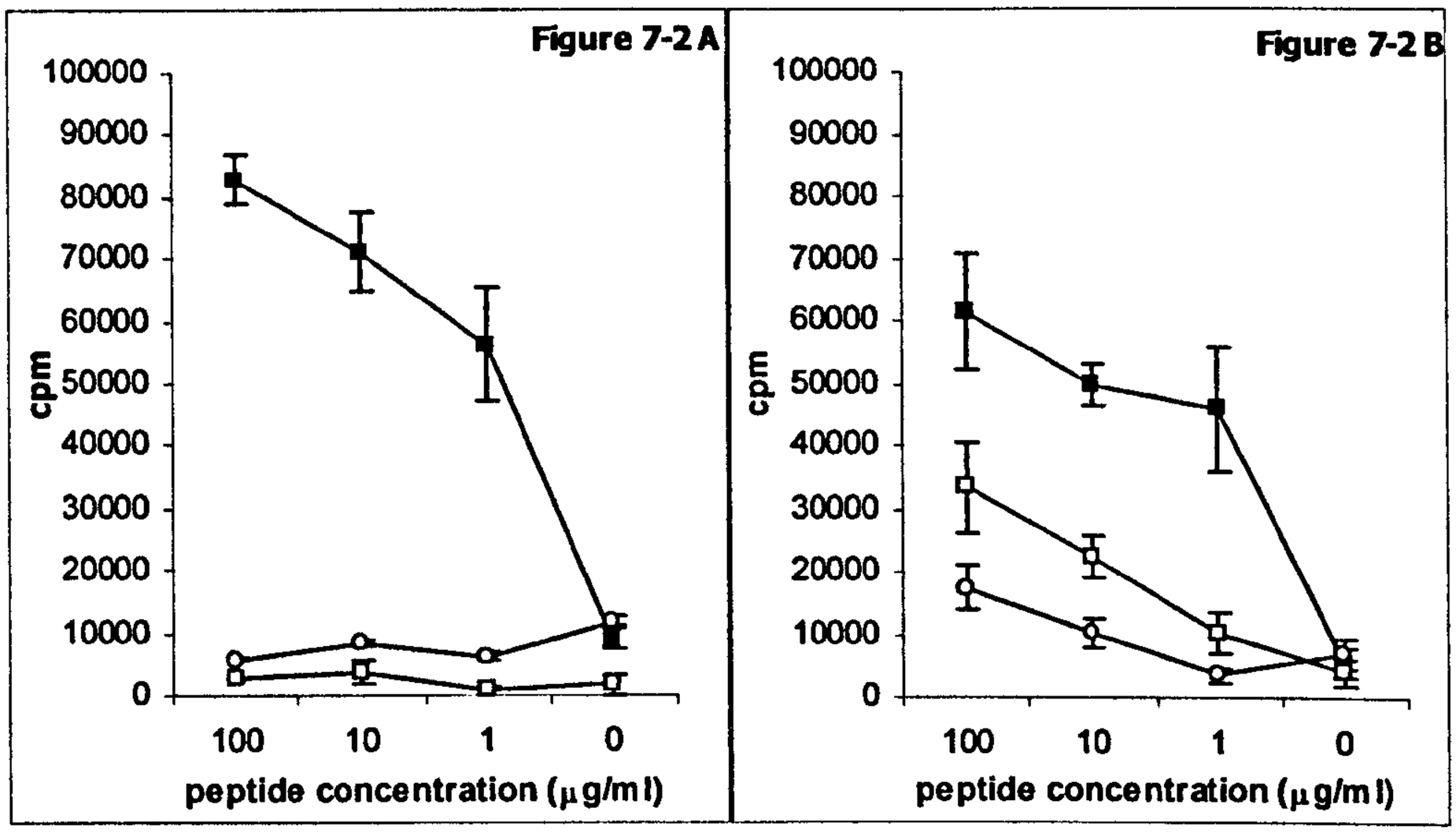


Figure 7-2: i.n. HBcAg vaccination; effect of LT adjuvant on $CD4^+$ proliferation

Groups of 5 C57/Bl6 mice were immunised with 1µg of HBcAg i.n with (■) or without LT (□). One group remained untreated(○). Ten days later mice were sacrificed and the proliferation of pools of B cell depleted cervical lymph node (Figure 7-2A) or spleen (Figure 7-2B) cells was measured by β-emission of cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of HBcAg peptide [aa 120-140] concentrations. Mean CPM of triplicate cultures of pooled cells are shown ± 1SD.

7.4 Intramuscular DNA Prime, Intranasal HBcAg Boost Vaccination

In line with the previous chapters, we aimed to determine the effect on DNA primed animals of boosting these same animals mucosally with purified HBcAg delivered i.n. An outline of the timings for the vaccination protocol is outlined in Figure 7-3.

7.4.1 HBcAg-Specific Humoral Responses

As observed previously, i.m. immunisation with pcDNA3.1/core induced a low but measurable response to the HBcAg in the sera of vaccinated mice (Figure 7-4). However, following i.n. boosting, this response was improved by approximately five-fold. This increase was found to be statistically significant and in contrast to the previous data, appeared to be independent of whether or not LT was included as a mucosal adjuvant ($p>0.95$ for both groups). In light of these data it is not clear whether the protein boost is responsible for the measured increase in titres observed or whether these increases are the result of continued expression from the pcDNA3.1/core vector. Although time did not allow completion of these experiments, it would be relatively easy to determine whether this was the case by examination of HBcAg titres from animals primed with pcDNA3.1/core only.

In contrast, animals primed with the vector alone showed only very modest levels of anti-HBcAg in the serum (in line with those responses described in 7.3.1). Disappointingly, no antigen specific antibodies against HBcAg or LT could be detected in any of the mucosal washes tested.

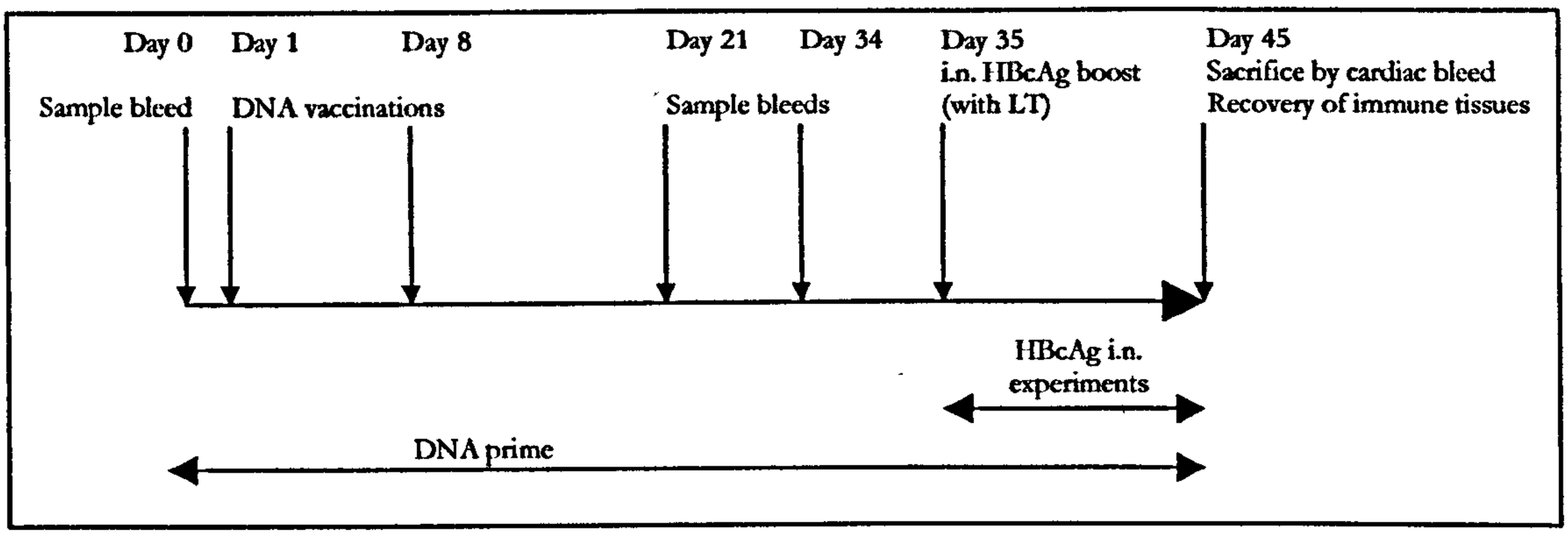


Figure 7-3: Vaccination regime for DNA prime, i.n. boost experiments

The timing and nature of vaccinations and sampling in the experiments described in this chapter.

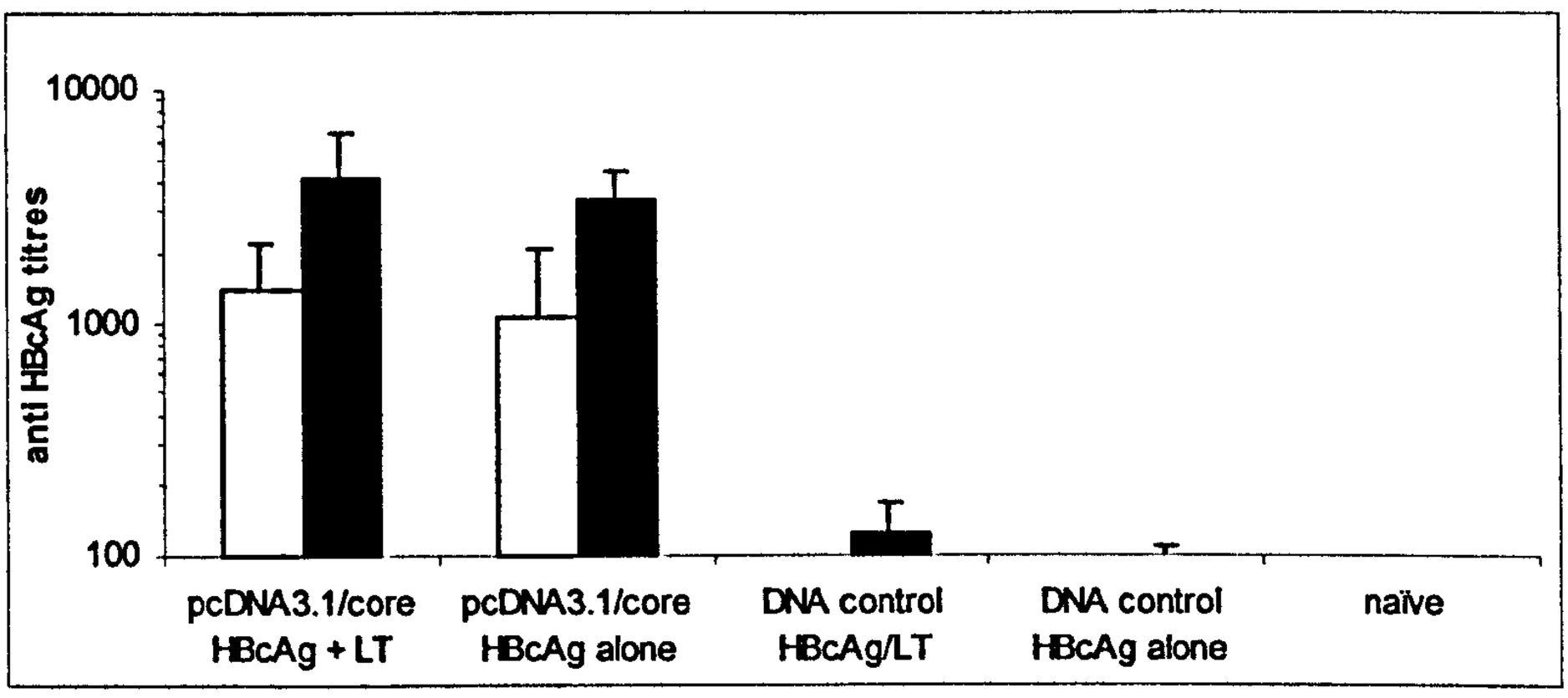


Figure 7-4: HBcAg-specific titres in the sera of DNA-primed mice pre- and post-boost vaccination

Groups of 10 C57/Bl6 mice were immunised i.m. with 100μg DNA (pcDNA3.1/core and DNA control groups), on days 1 and 8 of the experiment. 5 mice from each group were boosted with 1μg HBcAg with LT, 5 with HBcAg alone on day 35. The mean serum anti-HBcAg titres are shown on day 34 (□) and day 45 (■), ± 1S.D.

7.4.2 $CD4^+$ T Cell Proliferation

In addition to humoral responses, $CD4^+$ proliferative responses were also measured in the local lymph nodes and spleens of the i.m. vaccinated mice. Figure 7-5 shows the responses observed in the CLN of DNA-primed animals boosted with purified protein in the presence or absence of LT as an adjuvant. The responses in spleen cells are shown in Figure 7-6.

In contrast to expectations, in both local lymph nodes and splenic tissue, pre-immunisation with the pcDNA3.1/core vector appears to reduce the capacity of the mucosal adjuvant LT to stimulate $CD4^+$ specific T cells as responses in these animals were shown to be lower than those observed in animals that were immunised with vector alone. In these animals, $CD4^+$ cells from both local lymph nodes and spleens showed a very strong proliferative response following immunisation with LT and HBcAg (similar to that seen on immunisation with HBcAg and LT without any DNA prime, Figure 7-2). In addition, there is a consistency in the data with that observed previously in that proliferation was seen in lymph nodes when an HBcAg boost was given with LT, whilst in the spleens, proliferative responses were generated whether HBcAg was given alone or with LT.

The magnitude of the humoral response generated does not appear to be significantly different between the two groups. This would suggest one of two possible explanations. Either the humoral response is unaffected by the mucosal boost (antibodies measured are generated by DNA alone), or the key location for interaction between primed T-cells for enhancement of the immune response is the spleen.

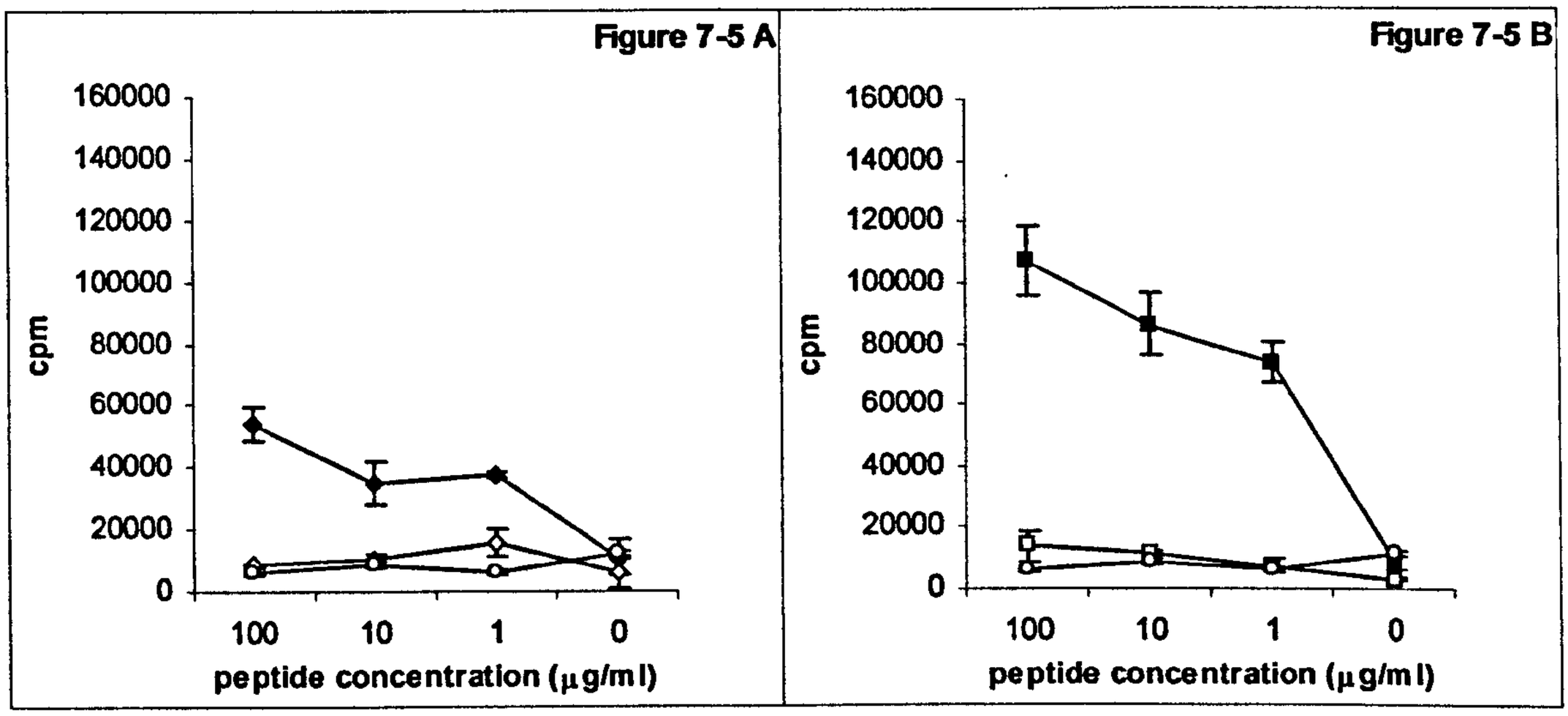


Figure 7-5: Measurement of HBcAg specific $CD4^+$ responses in the CLN of DNA-primed mice boosted i.n. with purified HBcAg

Groups of 5 C57/Bl6 mice were immunised as described in Figure 7-4: i.m. with 100µg DNA (pcDNA3.1/core and DNA control groups), on days 1 and 8 of the experiment. All groups were boosted with 1µg HBcAg, either with or without LT, on day 35. Ten days later mice were sacrificed and the proliferation of pools of B cell depleted lymph node cells was measured by β-emission of cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of HBcAg peptide [aa 120-140] concentrations. Mean CPM of triplicate cultures of pooled cells are shown ± 1SD. Figure 7-5A shows mice primed with pcDNA3.1/core and boosted i.n. with HBcAg and LT (◆) or HBcAg alone (◇). Figure 7-5B shows mice primed with control DNA and boosted i.n. with HBcAg and LT (■) or HBcAg alone (□).

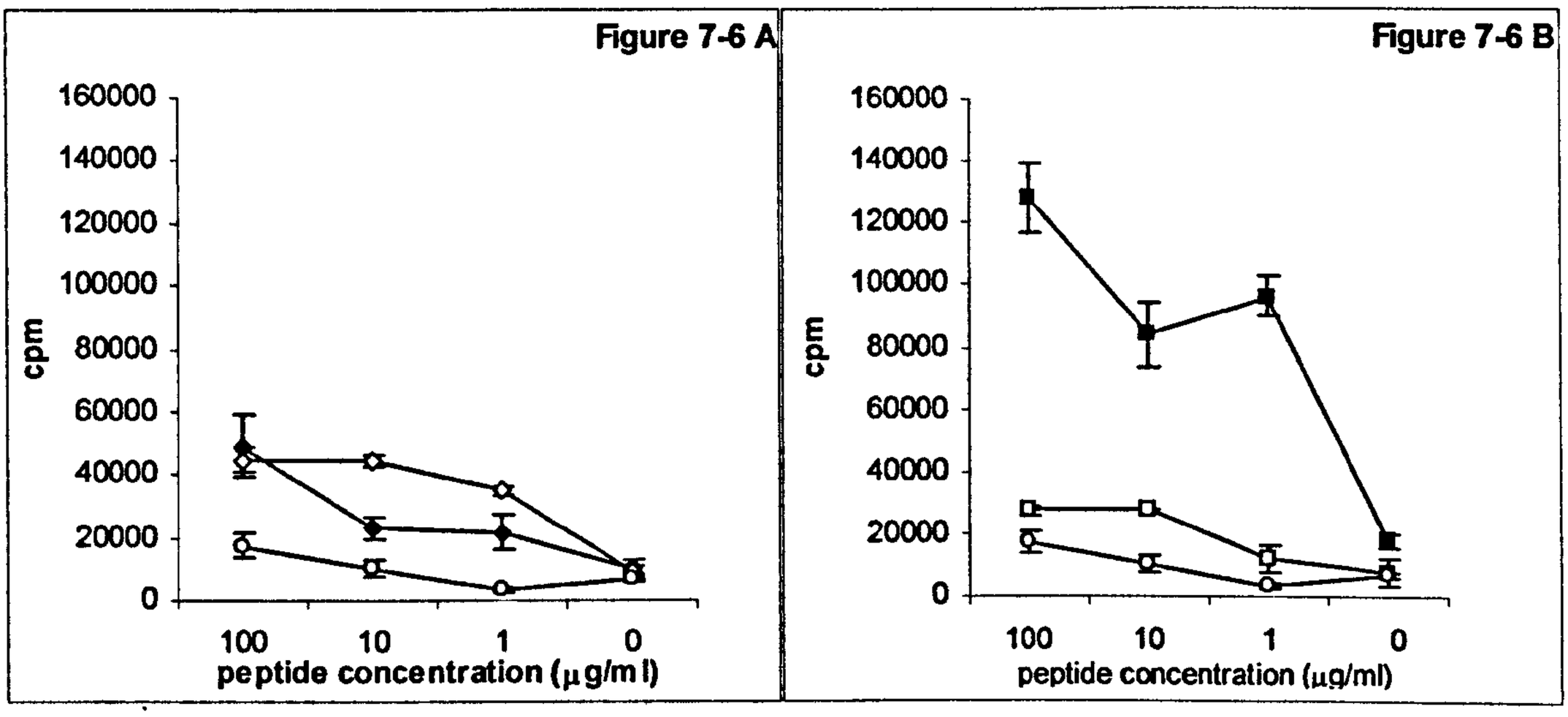


Figure 7-6: Measurement of HBcAg specific $CD4^+$ responses in the spleens of DNA-primed mice boosted i.n. with purified HBcAg

Mice were vaccinated and spleen cell proliferation measured in the same way as for lymph nodes (Figure 7-5). Figure 7-6A shows mice primed with pcDNA3.1/core and boosted i.n. with HBcAg and LT (◆) or HBcAg alone (◇). Figure 7-6B shows mice primed with control DNA and boosted i.n. with HBcAg and LT (■) or HBcAg alone (□).

7.5 Gene Gun DNA-Prime, intranasal HBcAg Boost Vaccination

7.5.1 HBcAg-specific Humoral Responses

As described previously, in response to the gene gun vaccination with pcDNA3.1/core a specific and detectable response to HBcAg was observed prior to boosting, as shown in Figure 7-7. On mucosal boosting with the purified protein and LT, the magnitude of this response was significantly enhanced over that observed prior to the boost ($p=0.00722$). There was also an increase in the group boosted with HBcAg alone, but this was increase was not statistically significant due to variation in individual titres within the group. As with the i.m. prime and boosted experiment, the inclusion of LT as the adjuvant did enhance the humoral response over that observed when the antigen was used alone. Once again, no IgA specific responses to HBcAg or LT could be detected in nasal or pulmonary washes.

7.5.2 $CD4^+$ T Cell Proliferation

The HBcAg-specific proliferations measured in response to this prime boost regimen are shown in Figure 7-8 (CLN) and Figure 7-9 (spleen) respectively. In the local lymph nodes, gene gun administration of pcDNA3.1/core followed by an i.n. boost of HBcAg alone resulted in proliferation of the same level as that induced by vaccination with control DNA boosted by HBcAg with LT. When animals were primed with pcDNA3.1/core and boosted with HBcAg with LT the response was higher still. This situation is the reverse of the observed following i.m. vaccination, where priming the animals with pcDNA3.1/core and boosting with LT + HBcAg appeared to reduce the proliferative response. Once more, the magnitude of splenic

responses did not appear to be affected by the inclusion of LT as a mucosal adjuvant.

These data are of particular interest in that the pattern of the response being enhanced at the local (lymph node) rather than the systemic (spleen) level following gene gun vaccination corresponds to that observed following systemic boosting of DNA primed animals as shown in Chapter 5.

7.6 Comparison of IgG Subtype Titres

Figure 7-10 shows a comparison of the IgG1 and IgG2a titres measured in response to the prime-boost vaccination regime with and without LT. In line with the total IgG titres, the IgG2a titres are higher in the i.m. group than the gene gun group. As previously noted in chapter 5, gene gun, but not i.m. vaccination, induced low levels of IgG1 antibody, indicating a more Th2 like response in the gene gun group. Inclusion of LT as a mucosal adjuvant in these experiments did not appear to influence the IgG1:IgG2a generated.

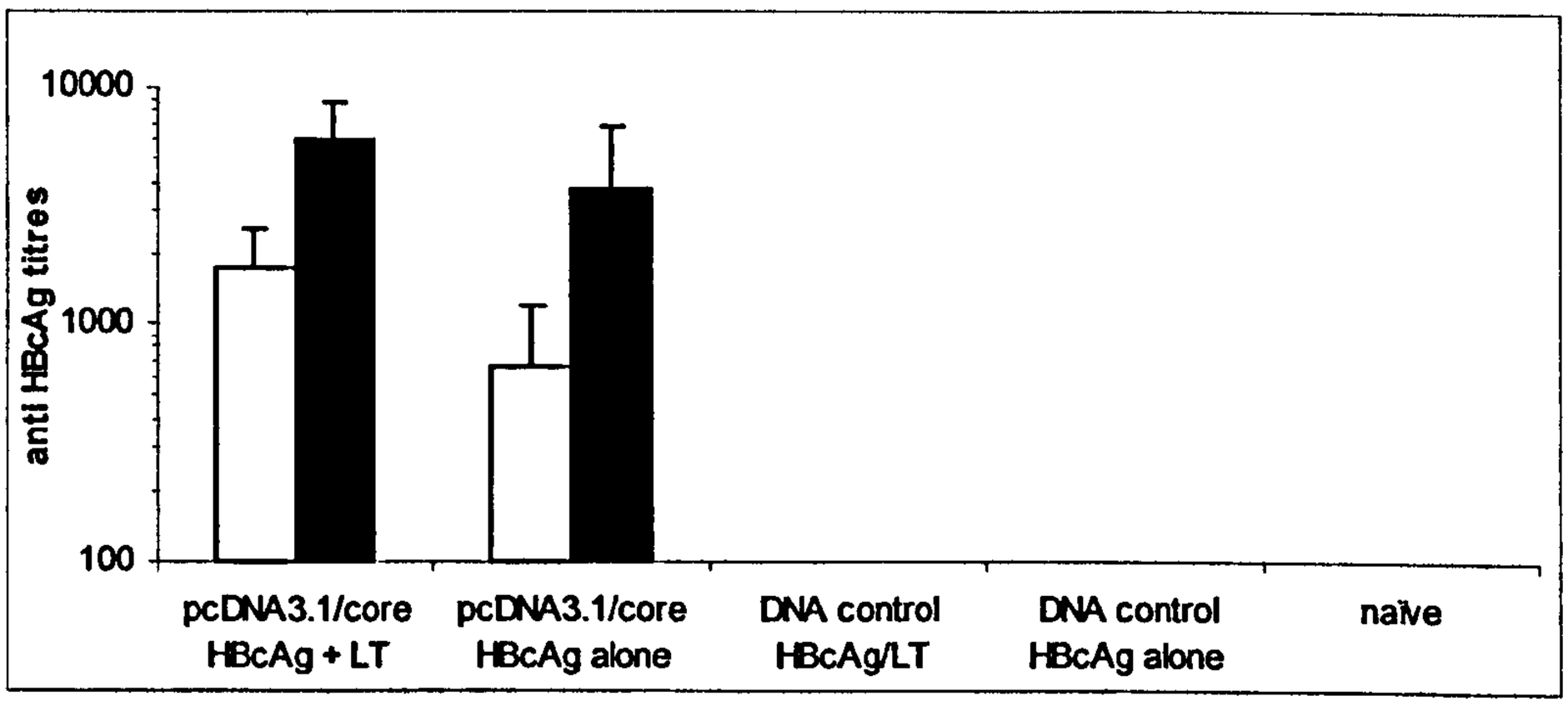


Figure 7-7: HBcAg-specific titres in the sera of gene gun DNA-primed mice pre- and post-i.n. HBcAg boost vaccination

Groups of 10 C57/Bl6 mice were immunised by gene gun with 100μg DNA (pcDNA3.1/core and DNA control groups), on days 1 and 8 of the experiment. 5 mice from each group were boosted with 1μg HBcAg with LT, 5 with HBcAg alone on day 35. The mean serum anti-HBcAg titres are shown on day 34 (□) and day 45 (■), ± 1S.D.

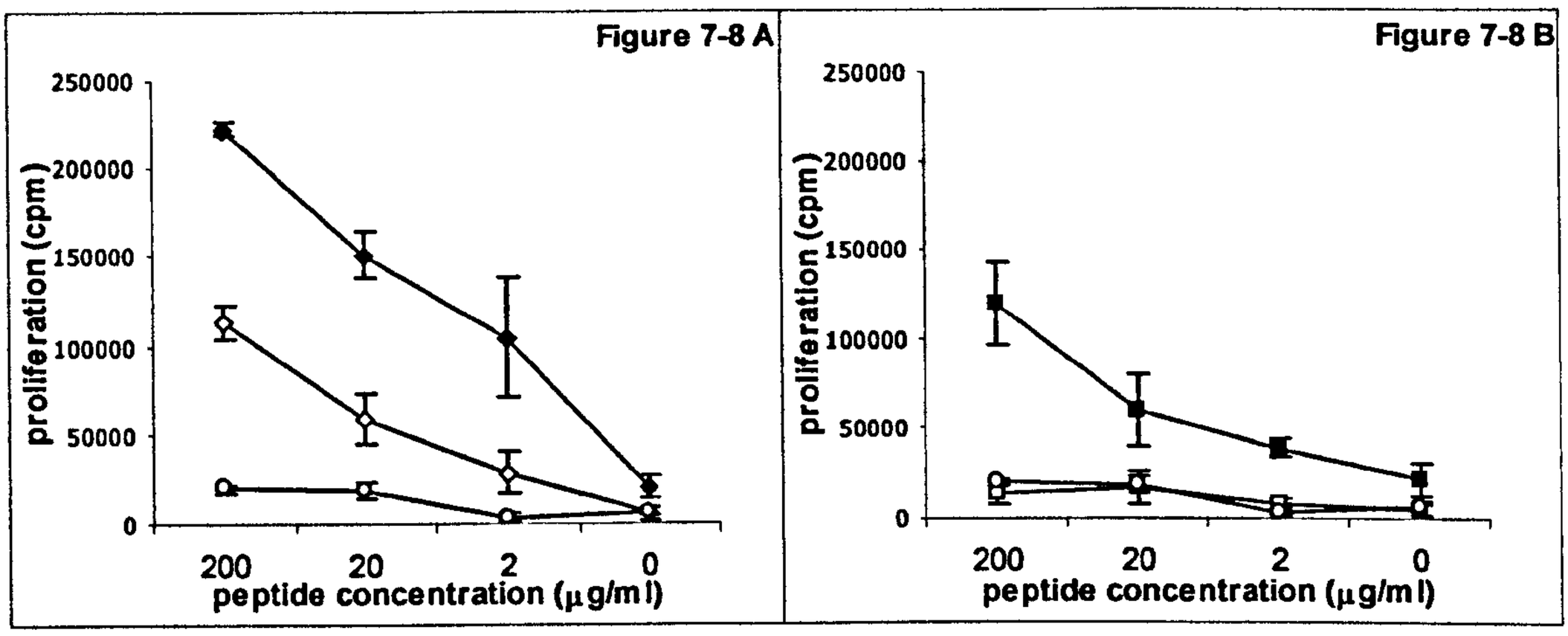


Figure 7-8: Measurement of HBcAg specific $CD4^+$ responses in the CLN of gene gun DNA-primed mice boosted i.n. with purified HBcAg

Groups of 5 C57/Bl6 mice were immunised as described in Figure 7-4: by gene gun with 100µg DNA (pcDNA3.1/core and DNA control groups), on days 1 and 8 of the experiment. All groups were boosted with 1µg HBcAg, either with or without LT, on day 35. Ten days later mice were sacrificed and the proliferation of pools of B cell depleted cervical lymph node cells was measured by β-emission of cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of HBcAg peptide [aa 120-140] concentrations. Mean CPM of triplicate cultures of pooled cells are shown ± 1SD. Figure 7-8A shows mice primed with pcDNA3.1/core and boosted i.n. with HBcAg and LT (■) or HBcAg alone (□). Figure 7-8B shows mice primed with control DNA and boosted i.n. with HBcAg and LT (◆) or HBcAg alone (◇).

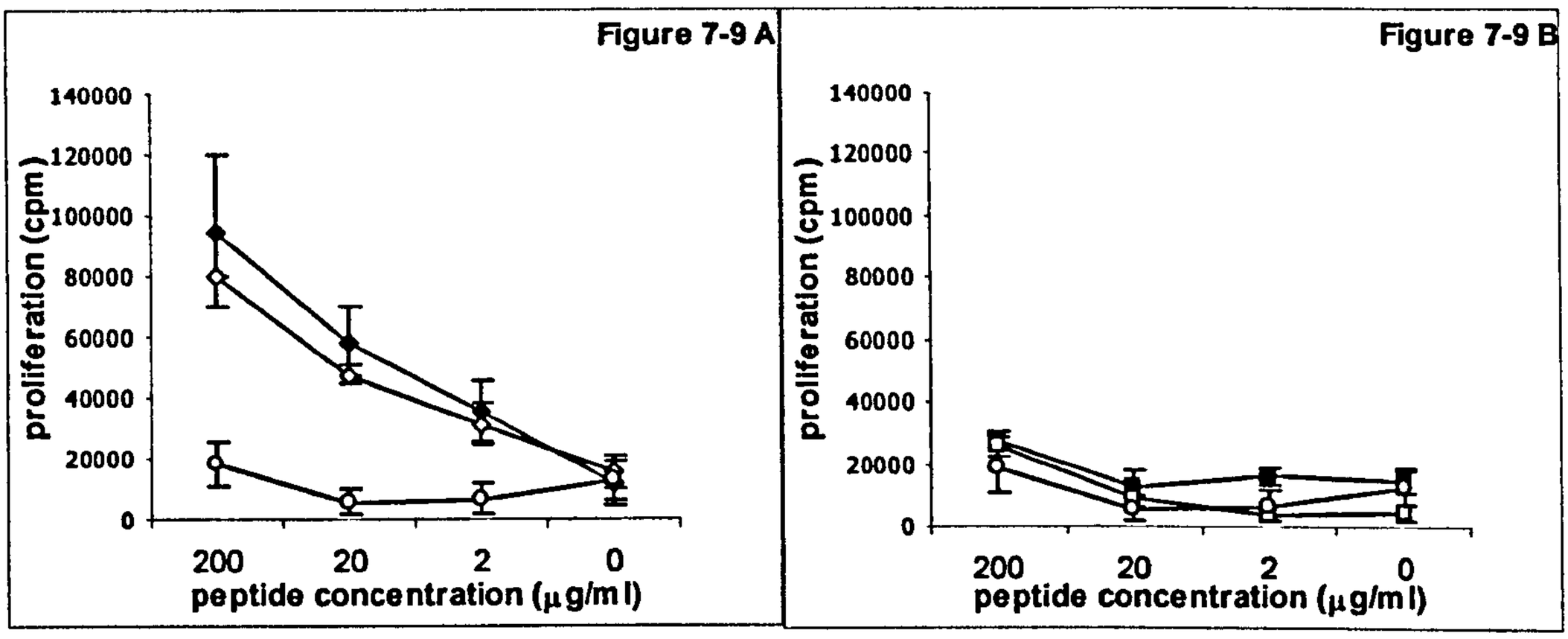


Figure 7-9: Measurement of HBcAg specific $CD4^+$ responses in the spleens of gene gun DNA-primed mice boosted i.n. with purified HBcAg

Groups of 5 C57/Bl6 mice were immunised as described in Figure 7-4: by gene gun with 100µg DNA (pcDNA3.1/core and DNA control groups), on days 1 and 8 of the experiment. All groups were boosted with 1µg HBcAg, either with or without LT, on day 35. Ten days later mice were sacrificed and the proliferation of pools of B cell depleted spleen cells was measured by β-emission of cells after DNA incorporation of [^{3}H] thymidine over 16 hours following 3 day restimulation with a range of HBcAg peptide [aa 120-140] concentrations. Mean CPM of triplicate cultures of pooled cells are shown ± 1SD. Figure 7-9A shows mice primed with pcDNA3.1/core and boosted i.n. with HBcAg and LT (■) or HBcAg alone (□). Figure 7-9B shows mice primed with control DNA and boosted i.n. with HBcAg and LT (◆) or HBcAg alone (◇).

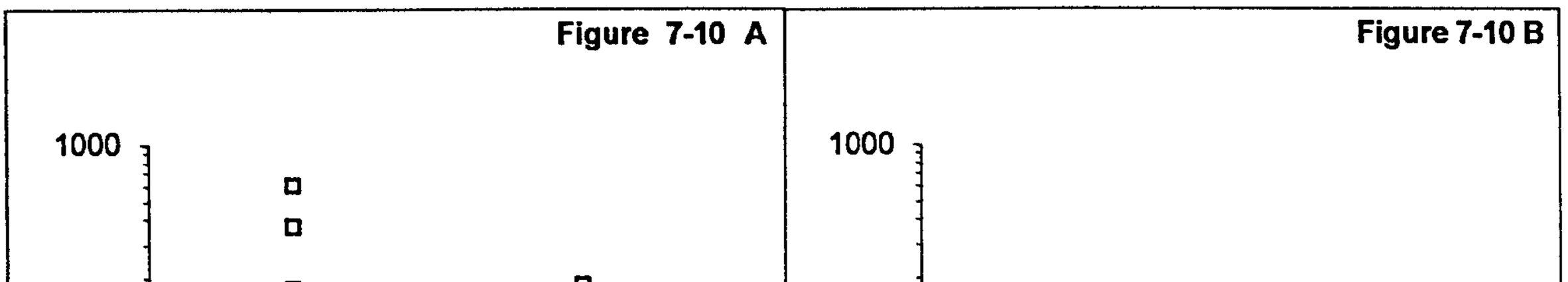


Figure 7-10: Antibody subtype titres following i.m. and gene gun vaccination.

C57/Bl6 mice were immunised with 100μg pcDNA3.1/core or control DNA on days 1 and 8, either i.m. (Figure 7-10A) or by gene gun (Figure 7-10B). Animals were boosted with 1μg HBcAg i.n., with or without LT on day 35. Immunoglobulin subtypes were examined on day 45. Calculated specific anti-HBcAg titres are shown. ▲ represent IgG1 titres for individual mice, □ represent IgG2a titres.

7.7 Mucosal Antibody Production

Given the strong $CD4^+$ proliferation observed in these experiments in local lymph nodes, it was hoped that a mucosal antibody response would develop. However, this promise was not borne out by ELISAs using nasal and pulmonary washes; no HBcAg specific IgA responses could be detected in any samples. To determine if a secretory antibody response had developed against the adjuvant, LT-specific ELISAs of these samples were also conducted, but again, no specific responses could be detected.

This was disappointing, and upon reflection, it may be that either a greater length of time is required between the delivery of the protein boost and the examination of the mucosal tissues, or that multiple doses of LT and HBcAg are required to maximise this response. In previous studies, antigen specific IgA has been observed in nasal and lung washes following at least three immunisations with a similar toxin (Douce *et al.* 1997).

7.8 Summary of Results

7.8.1 Humoral Responses

Overall, the magnitude of the humoral responses generated when animals are primed i.m. or by gene gun and boosted mucosally are remarkably similar. Final titres, after the boost, are slightly higher in the gene gun experiments when equivalent groups are examined, but these differences are small and not statistically significant. Disappointingly, neither vaccination strategy resulted in measurable IgA specific antibodies against either HBcAg or against LT.

7.8.2 $CD4^+$ T Cell Responses

The experiments described in this chapter demonstrate that there are differences in the localisation of $CD4^+$ specific T-cells following priming with DNA either by i.m. or gene gun delivery. As seen before in Chapter 5, following boosting of gene gun immunised animals, the majority of proliferation occurs in the lymph nodes, whilst in animals vaccinated i.m., the greater responses were found in the spleen following the boost. However, the use of LT appears to introduce a second location-specific response. Since it is a mucosal adjuvant, it is unsurprising that the toxin enhanced the responses observed in the CLN, however, more interesting is the fact that these responses were enhanced in these lymph nodes if the animals were primed using pcDNA3.1/core using the gene gun. In contrast use of LT in mice primed using the i.m. route generated less proliferation in the local lymph nodes than those immunised with the vector backbone. The implications for these observations on use of prime boost strategies are given in the Discussion.

8 DISCUSSION

Although there has been a large volume of work conducted on the efficacy of DNA vaccination, there have been few studies systematically comparing the delivery of one antigen by different vaccination strategies. Likewise, although prime-boost vaccination has been studied in depth, parallel studies comparing delivery of a particulate antigen by different prime-boost vaccination strategies have rarely been conducted, in fact, only one study (based on a regime of DNA-prime, protein boost (Schirmbeck and Reimann 2001)), could be found. The results presented in this thesis directly compare the immune response to i.m. and gene gun HBcAg DNA vaccination in three prime-boost vaccination strategies. The strategies studied were: systemic DNA vaccination boosted either systemically with (i.m.) recombinant protein, or mucosally using purified protein or attenuated *Salmonella* strain as a delivery vehicle.

8.1 Intramuscular DNA Vaccination

In order to examine the impact of vaccinating with the DNA vaccine pcDNA3.1/core alone, the response to i.m. delivery of the plasmid was studied in isolation. These experiments are described in Chapter 5. Several experiments consistently showed the ability of the plasmid to induce a limited humoral response. The magnitude of this response was found to be independent of whether DNA was delivered i.m. or by gene gun. The generally low level of antibody produced by DNA vaccination when unaccompanied by a heterologous boost is probably a consequence of too low a concentration of protein in the extracellular environment

for the activation of significant numbers of B cells. This is consistent with work described in the literature describing variable humoral responses to DNA vaccination (Davis and McCluskie 1999).

$CD4^+$ T cell proliferation was not observed over the short time-scale initially examined, when DNA was given on days 1 and 8 and cells recovered on day 21. However, an alternate protocol in which mice were immunised with two doses of DNA on days 1 and 28, and cells were examined on day 56, revealed strong proliferation. This shows the importance of selecting the most appropriate time-scale for vaccination and examination of responses.

In contrast, a strong $CD8^+$ response could be measured relatively quickly (10 days) after a single vaccination. These could be restimulated *in vitro* with an HBcAg specific peptide to secrete IFN-γ and were also shown to be capable of killing target cells expressing an HBcAg epitope in an antigen-specific manner. These results are in agreement with a body of work in the literature, regarding activation of $CD8^+$ T cells following DNA vaccination, as measured by IFN-γ secretion (Sedegah *et al.* 1998), (not previously shown for HBcAg), and antigen-specific cytotoxicity in the same cells (which has been shown in HBcAg studies: (Bohm *et al.* 1996; Kuhrober *et al.* 1996). The relatively early time point at which these can be measured suggests that expression of antigen in the context of MHC class I occurs more rapidly than in the context of MHC class II.

Although vaccination with pcDNA3.1/core alone demonstrated that this vaccine has the potential to induce powerful cellular responses, the low humoral response generated reduces the applicability of these type of vaccines over a greater number of diseases in which both cellular and humoral responses are important. Therefore the challenge remained of strengthening humoral responses and perhaps of enhancing the existing cellular responses. The experiments discussed below (and

detailed in the remainder of Chapter 5, and Chapters 6 and 7), examined the heterologous prime-boost strategies that were studied in order to achieve these aims.

8.2 DNA Prime, Recombinant Protein Boost Vaccination

Animals that had been primed with the pcDNA3.2/core construct were boosted with small amounts of purified antigen alone (as outlined in the latter part of Chapter 5). These experiments consistently showed that the humoral responses primed by pcDNA3.1/core could be enhanced by boosting the animals with purified protein. These responses were significantly higher than those titres observed in animals which were only given the purified protein boost or were pre-immunised with the vector backbone alone. In addition, cellular responses did not appear to be affected following i.m. boosting with recombinant protein. The $CD4^+$ Th proliferations induced were equivalent to those seen following multiple vaccinations with the protein, in line with observations in the literature (Böcher *et al.* 2001).

Unfortunately, results from later experiments, in which DNA alone was administered and responses were measured after a longer time period, did not determine whether the strong $CD4^+$ response measured following prime-boost vaccination results from the boost effect. It could also be due to continual exposure of the antigen from the DNA vaccine over a long period of time, as seen in the extended time-scale DNA alone experiment. It seems likely, though, that both the prime and booster vaccinations contribute to the $CD4^+$ response, since the purified HBcAg protein has been shown to elicit such responses following i.m. injection of the purified antigen alone (Milich and McLachlan 1986).

The responses to i.m. and gene gun vaccination appeared superficially similar, but two main differences were observed. These were: firstly, the localisation of the $CD4^+$ cells that exhibited the strongest HBcAg-specific proliferation and secondly, the pattern of antigen-specific IgG subtype production. Intramuscular vaccination with DNA and recombinant protein induced strong $CD4^+$ proliferative responses in cells isolated from the spleen, but weaker responses in draining lymph nodes close to the site of vaccination, at the time-point examined. Interestingly, this observation is reversed with epidermal gene gun vaccination; higher $CD4^+$ proliferation was observed in the lymph nodes than in the spleen. This may be linked to the timing of the experiments; it has been shown that T cell responses initiate in draining lymph nodes and are not observed until later in the spleen (Akbari *et al.* 1999). Alternatively it may suggest that less antigen is being generated using this route of immunisation and may reflect the lower dose of DNA used for this form of vaccination.

It has been suggested that activation of naïve $CD4^+$ cells depends solely upon antigen presentation by DCs (Akbari *et al.* 1999; Jenkins *et al.* 2001). In the case of gene gun vaccination, the main group of cells involved would be LCs, a subset of DCs that form an almost continuous layer in the dermis, and which are directly transfected during gene gun vaccination (Tighe *et al.* 1998). These cells become migratory due to inflammatory signals caused by tissue damage associated with vaccination and express chemokines, which direct migration to the secondary lymphoid tissues. Within these tissues they are able to activate $CD4^+$ T cells following specific recognition of the presented antigen by the TCR (see Introduction section 1.4.1.2). The occurrence of a stronger $CD4^+$ proliferation in the lymph nodes following gene gun vaccination could therefore also be due to the

preferential initial migration of the DCs transfected during vaccination to the lymph nodes prior to the spleen (Jenkins *et al.* 2001).

Following i.m. DNA vaccination, on the other hand, myeloid DCs (from other sites within the body) rather than DCs will be involved in the processing and presentation of antigen to $CD4^+$ cells. These DCs will be attracted to the site of vaccination due to inflammation caused by tissue damage. They could process and present HBcAg epitopes as MHC class II-peptide complexes by one of two mechanisms. Firstly by taking up HBcAg, expressed by myocytes, as exogenous antigen (which is the same mechanism by which purified HBcAg from the boost vaccination is processed and presented). A perhaps less likely mechanism, would be that the DCs internalise the pcDNA3.1/core plasmid and express the HBcAg themselves. As well as the MHC class I presentation that would follow expression, it is possible that some peptide would be presented by MHC class II through cross-priming mechanisms (see Introduction section 1.4.1.3). Whichever mechanism is responsible, the DCs may then migrate directly to the spleen rather than to peripheral draining lymph nodes, which could explain the higher $CD4^+$ proliferation observed in the spleen following i.m. DNA immunisation (Akbari *et al.* 1999; Bajenoff and Guerder 2003). Further details of the localisation of the T cell response are discussed below in section 8.6.

Another difference between the responses following i.m. and gene gun vaccination is nature of the humoral response. These results indicate that both i.m. DNA vaccination with a plasmid encoding HBcAg and vaccination with purified protein, generate a strong IgG2a response, with little or no IgG1. In contrast, gene gun vaccination results in response that includes both subtypes of antibody. This mixed response is in contrast to previous work conducted in this laboratory and elsewhere (Zlei 2002; Zhou *et al.* 2003), showing a more definitive shift to a Th2

phenotype when DNA vaccines encoding non-particulate antigens (Tetanus toxin fragment C) are delivered by gene gun. It is therefore possible that the differences observed reflect the impact of the structure of the particle on the response induced. For example, it has previously been shown that the HBcAg preferentially induces Th1 responses when delivered i.m., as indicated by the presence of IgG2a, but no IgG1 anti-HBcAg antibodies. This response is believed to reflect the particulate nature of the antigen, as mice immunised with HBeAg, (an HBV protein with near identical sequence but which does not polymerise into a particulate form) exhibited a response dominated by IgG1 (Milich *et al.* 1997).

This result suggests that the antigen is being expressed following i.m. vaccination that it is being generated in sufficient quantities to generate particulate structures, (although there is no direct evidence of this). In contrast, the mixed response observed following gene gun vaccination (which usually induces Th2 responses (Zhu *et al.* 2004) suggest that that some particle structure is also being generated. However, due to the relatively low amount of DNA delivered and therefore protein expressed, the concentration maybe insufficient for generation of high numbers of complete particles leading to the mixed response observed. Therefore, gene gun delivery of pcDNA3.1/core may still have potential for immunomodulation of existing Th2 responses to Th1, unlike delivery of other antigens by gene gun (see Introduction section 1.5.4.3) and section 8.6 on therapeutic vaccination, below.

Trans-dermal protein vaccination, like gene gun vaccination, induces Th2 biased responses, as determined by cytokine production and antibody subtype analysis (Strid *et al.* 2004), whereas i.m. DNA vaccination gives Th1 responses (Feltquate *et al.* 1997). This suggests that it is the route of vaccination that results in the induction of Th2 cells rather than the nature of the vaccine (DNA or protein).

It is likely that LCs are the most important APCs involved in generation of the responses in these cases, given the presence of large numbers of LCs in the dermis. Myeloid derived DCs (MDC) in mice express TLR-9 and can therefore their antigen presenting and T cell activating capabilities can be enhanced by CpG motifs (Cella *et al.* 1999; Krug *et al.* 2001). Most MDCs, such as those derived from peripheral blood monocytes, preferentially induce $CD4^+$ differentiation into Th1 cells (Rissoan *et al.* 1999). However, the LCs that reside in the dermis, although derived from the same lineage, have been shown to differ in several aspects from other MDC, including a lack in IL-12 production (a pro-Th1 cytokine) following stimulation (Peiser *et al.* 2004). Since vaccine antigen is more likely to encounter other MDCs capable of producing IL-12, this could explain the resultant Th2 bias of gene gun vaccination compared to i.m. vaccination.

Another potential factor in the bias of the $CD4^+$ response is the level of CpG motifs present in the two forms of vaccination. Although both methods produce sufficient antigen to stimulate cellular and humoral responses, i.m. vaccination uses greater quantities of DNA, and therefore contains more CpG motifs. Since these motifs preferentially generate Th1 immunity (see Introduction section 1.5.4.3) this provides a simple potential explanation for the results, backed up by observations that addition of CpGs to gene gun delivered DNA overcomes the usual Th2 bias of this strategy (Schirmbeck and Reimann 2001; Zhou *et al.* 2003).

8.3 DNA Prime, Oral *Salmonella* Boost Vaccination

Several prime-boost studies involving delivery of attenuated *Salmonella* expressing vaccine antigens have been performed to date. In one such experiment

Salmonella expressing a *Mycobacterium tuberculosis* antigen were delivered by i.v. injection to animals that had been primed by gene gun with DNA expressing the same antigen (Mollenkopf *et al.* 2001). Interestingly, whilst the *Salmonella* expressing the antigen offered some protection against *M. tuberculosis* challenge, the animals which had been primed with DNA did not experience enhanced protection. Unfortunately this study failed to characterise specific immunological readouts and it is therefore unclear why the DNA prime appeared ineffective. Other prime-boost vaccine strategies involving *Salmonella* have in general used the bacteria as the prime immunisation (Devico *et al.* 2002; Londono-Arcila *et al.* 2002). In these studies, *Salmonella* prime was followed by a heterologous boost using purified protein. As such it is difficult to directly compare the results with the work in this thesis, although these experiments show that the prime-boost strategies offer improved immune responses over single vaccinations.

One recent study found that boosting gene gun primed BALB/c mice with *Salmonella* gave a much more Th1-like response than mice given the gene gun prime alone (Lange *et al.* 2004). This provides some evidence that gene gun delivery might not preclude the use of a strategy as a therapeutic vaccine designed to initiate a type 1 response. However, it was not clear from this study whether this response would have been further enhanced if the mice had been initially primed using the i.m. route of immunisation.

The work presented in Chapter 6 explores the impact of priming the immune system using pcDNA3.1/core, by either i.m. injection or gene gun delivery and boosting with subsequent oral delivery of *Salmonella* expressing the same antigen.

The success of prime-boost strategies is dependent on both forms of vaccination being efficacious and synergistic. In these experiments, BRD509 pGA-

1 was able to generate serum antibody titres to HBcAg following i.m. priming with DNA. This indicates that there is at least some degree of positive interaction between the responses to two vaccines. This could take the form of restimulation of memory T cells from the DNA vaccination by *Salmonella*-expressed HBcAg presented by APCs either at the mucosa or elsewhere.

Interestingly, although a strong increase in antibody titres was observed following the *Salmonella* boost in the i.m. experiment, this increase was not observed when the DNA vaccine-prime was delivered by gene gun. A possible explanation for the observed difference involves the restimulation of memory $CD4^+$ T cells that had been activated in response to the initial DNA vaccination. For this restimulation to occur, HBcAg must be transported (free, expressed by *Salmonella* or carried by APCs) from the mucosa to locations where primed Th cells will be found (e.g. the spleen or peripheral lymph nodes). Evidence discussed elsewhere in this thesis suggests a localisation of the Th response in the spleen following i.m. DNA vaccination. If the *Salmonella* response was likewise directed to the spleen, this could enhance the existing response. However, in the case of a gene gun prime, the responses to the prime and boost would occur in separate locations and might not enhance antibody production. It is also possible that by using the different routes of DNA priming that antagonistic immune responses are produced. For example it is possible that the Th2 cells activated by the gene gun prime immunisation (Creusot *et al.* 2001) down-regulate responses to HBcAg expressed by *Salmonella*, which is known to generate a more Th1-like response (Medina and Guzman 2000).

Another interesting observation in the i.m. experiment was the complete lack of antibody response when previously naïve mice were immunised with BRD509 pGA-1, compared to a response in the group of mice primed with the control plasmid before boosting with the same bacteria. This result may reflect the

impact of unmethylated CpG motifs on the immune system. Pre-immunisation with pcDNA3.1 may have activated the immune system via interaction with cells bearing TLR-9, resulting in the upregulation of co-stimulatory molecules by APCs and providing the secondary signal needed in addition to specific antigen-recognition for the activation of naïve lymphocytes (see Introduction section 1.4.1). The plasmid expressing HBcAg also contains these motifs and therefore should help stimulate a specific antibody response following BRD509 pGA-1 vaccination in the same way as the control plasmid. In the parallel experiments using the gene gun, there was no significant difference in titres following the boost, whether animals were primed with control DNA or pcDNA3.1/core (Figure 6-5). This lack of response could reflect the failure of HBcAg-specific memory T cells and CpG-stimulated APCs to migrate to areas in which *Salmonella* expressing HBcAg are present. This theory is supported by the previous data that showed that i.m. DNA vaccination induced strong proliferative responses by cells from the spleen, whilst in contrast following gene gun immunisation proliferation was stronger in the lymph nodes than the spleen. In addition, gene gun delivery requires substantially lower amounts of DNA to prime a response, therefore there is less CpG DNA available for stimulation of Toll receptors. The impact of these motifs on the boost response could be further elucidated by the comparison of experiments in normal and TLR-9 knock-out mice. It is unclear at this stage the failure of gene gun control DNA to enhance future responses could be overcome by more appropriate timing of the delivery of the prime and boost or whether one of the limitations of DNA vaccination using the gene gun is lack of non-specific immuno-potentiation due to relatively small quantities of CpG motifs.

The inability to measure cellular responses from the vaccination experiments due to bacterial growth in *ex vivo* cultures was frustrating, given the added insight

that the data could have provided into the nature of the overall immune response. As well as the information on $CD4^+$ proliferation that would have helped to shed light on the development of the humoral responses, it would have been of interest to know the level of $CD8^+$ T cell activation.

The attenuated strain used in these experiments, *S. typhimurium* BRD509 has been studied previously with the same plasmid expressing an altered form of HBcAg (Londono *et al.* 1996). In these experiments, and in contrast to ours, bacteria were given twice, on days 1 and 56, without any prior DNA immunisation. This group also found that specific serum-antibody titres were observed against *Salmonella* and low responses were also detected to the HBcAg. It is therefore surprising that serum anti-HBcAg antibodies could not be measured following vaccination with *S. typhimurium* BRD509 pGA-1 alone. However, this experiment varied in two aspects; the first being the fact that two doses of *Salmonella* were used prior to measurement of any immunological parameter, the second being the fact that these experiments were originally carried out in BALB/c mice, an inbred strain which is particularly sensitive to *Salmonella* infection. In contrast, our experiments were carried out in C57/Bl6 mice to allow direct comparison with other experiments outlined in this thesis. Experiments subsequently carried out in both these mice strains indicated that although the *Salmonella* were maintained in higher numbers in the C57/Bl6 mice, the anti-LPS and anti-HBcAg titres were higher in the BALB/c animals. Therefore the development of the immune response is not solely dependent on longevity of the bacteria *in vivo*, but may also reflect the ability of the mouse strains to effectively process the bacterial antigens. In addition, these experiments showed that few bacteria recovered from tissues were expressing the HBcAg. A consequence of this result is the limitation placed on the potential of the *Salmonella* to boost any response primed by the DNA vaccination.

The lack of mucosal response to HBcAg following the BRD509 pGA-1 vaccination was also disappointing, although anti-*S. typhimurium* titres were detected. Again, an extended time-scale or repeat vaccinations might be of benefit under these circumstances.

Maximal synergy of the prime and boost vaccinations depends on the enhancement of APCs and lymphocytes specifically activated by the DNA vaccine being stimulated in the boost vaccination. This may include upregulation of activation signals in previously naïve cells, and the restimulation of memory T and B cells. One way of enhancing these effects would be to physically bring these cells into closer proximity. The simplest way this could be achieved would be to alter the sites of immunisation selected. Intra-peritoneal vaccination with DNA might result in stimulation of cells closer to the intestinal mucosa, however, this route has been shown not to generate antibody and CTL responses with viral antigens (Bohm *et al.* 1998). Similarly, *Salmonella* can be delivered by other routes of immunisation, for example intravenously. However, immunisation using this route completely ablates the potential induction of a mucosal response.

Despite the apparently better qualities of *nirB*, as a promoter in attenuated intracellular bacteria described in the literature (see Introduction section 1.5.3.5) both *htrA* (Roberts *et al.* 1998) and *pagC* (Dunstan *et al.* 1999) have been shown, in direct comparisons with *nirB*, to give higher antibody titres *in vivo*. Therefore, despite *in vitro* and even some *in vivo*, evidence, switching promoters to either *htrA* or *pagC* may have yielded improved immune responses.

8.4 DNA Prime, Intranasal Protein Boost Vaccination

Previous studies have shown that parenteral protein immunisation can prime mucosal immune responses, despite the fact that the sites of vaccination are in two separate immunological compartments (McCluskie *et al.* 2002). The experiments in Chapter 7 describe a similar approach in which the prime consists of a DNA vaccine followed by an intranasal protein boost either with or without the mucosal adjuvant LT.

The humoral responses of gene gun and i.m. DNA primed animals to mucosal boosting with purified antigen were found to be remarkably similar in these experiments. Although final antibody titres were slightly higher in gene gun experiments, these differences are small and not statistically significant. The important difference in the humoral response is the antibody subtype pattern, which shows a more Th1-like pattern (no IgG1 production) in the i.m. immunised mice, as previously observed in Chapter 5.

One of the aims of using a mucosal boost was the potential to additionally induce the production of antigen-specific secretory IgA that could be measured in nasal or pulmonary washes. However, none of the strategies using i.n. delivery of protein resulted in measurable titres. This was disappointing, but perhaps not surprising given the low systemic antibody response. Repeated vaccination or increased doses of HBcAg might give rise to production of sIgA. Interestingly there was no antibody response to LT in mucosal washes, despite its known efficacy as a mucosal adjuvant and the impact it was observed to have on $CD4^+$ responses. It is unclear why this occurred and may reflect the fact that most previous studies have looked for these responses following more than one dose of the toxin.

In terms of $CD4^+$ proliferation, inclusion of LT, led to very strong responses in the CLN, which were absent when HBcAg was given alone. In contrast, responses to HBcAg were observed in the spleen and inclusion of LT enhanced this response. This suggests that HBcAg delivered i.n. is not normally processed by APCs and T cells in the CLN without the presence of the LT.

When mice were primed with DNA prior to the i.n. administration of HBcAg, the pattern changes. The results shown in Chapter 5 and 7 indicate that, at least at the time-points examined, $CD4^+$ proliferation following gene gun vaccination occurs largely in the lymph nodes whilst following i.m. DNA vaccination it is mainly restricted to the spleen. A proliferative response occurs in the CLN following gene gun vaccination and i.n. boosting, which is interesting since the lymph nodes here are not physically close to the site of initial vaccination. This suggests a migration of HBcAg presenting APCs to distant (as well as local) lymph nodes following gene gun vaccination, or more likely, the trafficking of activated HBcAg-specific $CD4^+$ T cells through different lymph nodes. A slightly lower response was observed in the spleen, this may reflect the enhancement of an initial low level response to the gene gun vaccination by the i.n. boost.

This localisation had an interesting effect on the responses of mice to a boost containing LT. When mice primed by gene gun vaccination were boosted i.n. with HBcAg and LT, the CLN response was increased and spleen responses were unaffected, as might be expected from a mucosally active adjuvant. The most interesting effect, however, was the nature of the response generated when an i.m. DNA prime was used in conjunction with the HBcAg and LT boost. As noted above, i.m. DNA vaccination appears to induce strong splenic $CD4^+$ T cell proliferation. The presence of LT in the boost had no effect on the splenic proliferation, but did result in a measurable response in the CLN (compared to no

response without LT). However, the responses in both the spleens and CLN of mice primed with control DNA were much higher. It appears that priming the mice with pcDNA3.1/core by the i.m. route reduced the ability of mice to respond to a boost containing LT.

One possible explanation for this is that HBcAg-specific T cells migrate to (or are maintained in) the spleen following i.m. vaccination. These activated cells, would not respond to chemokine signals attracting naïve T cells to the site of action of LT, thus preventing the development of a CLN response (Sallusto *et al.* 2004). Alternatively, hyper-stimulation of previously activated T cells could result in apoptosis of a proportion of the HBcAg-specific population. Whatever the explanation, immunisation by gene gun is clearly the more appropriate method for priming immunity when an intranasal boost containing LT – and possibly other mucosal adjuvants – is considered.

It was interesting that strong, if localised $CD4^+$ T cell proliferation was accompanied by only poor antibody responses. These observations could be reconciled by considering the time-scale over which responses develop following vaccination. Following the first exposure to an antigen, an antibody response takes approximately 7 days to initiate, and then increases until it reaches its maximum level at about 21 days post immunisation, unless subsequently boosted. Therefore at 10 days after immunisation the response will be far from its maximum level. On the other hand, $CD4^+$ T cell proliferation in response to an antigen is at its maximum between 9 and 15 days after immunisation. Given the strength of the $CD4^+$ response and the timing of the experiment, it is likely that the antibody response would continue to increase with time.

The poor mucosal immunogenicity of the purified protein alone may reflect several factors that control the responses at mucosal surfaces. Many antigens do

not stimulate immune responses when presented at mucosal surfaces, instead inducing a state of tolerance (Eriksson and Holmgren 2002). However, particulate antigens have been shown to be effective mucosal adjuvants, therefore repeated or increased doses of HBcAg may have led to stronger humoral responses. In fact, it has been shown that a chimeric HBcAg particle expressing a heterologous influenza virus peptide inserted into the c1 loop was able to generate antibody-mediated protection against challenge with a lethal dose of influenza virus following i.n. immunisation (Neirynck *et al.* 1999).

The vaccination strategy of i.m. pcDNA3.1/core coupled with an i.n. boost of purified HBcAg and LT as an adjuvant was successful in enhancing the humoral and cellular immune responses generated by the pcDNA3.1/core alone. In agreement with previous experiments in this thesis the specific antibody response indicated that the majority of $CD4^+$ T-cell help was from cells of the Th1 phenotype.

8.5 Mechanisms for Localisation and Biasing of Cellular Immune Responses

The results shown in Chapters 5 and 7 indicate that $CD4^+$ T cell responses are at least partially localised to either the spleen or local lymph nodes following, respectively, i.m. or gene gun DNA vaccination. Whilst accepting that this may reflect timing of the experiments undertaken and the influence of the route of both priming and boosting vaccinations, these observations may have practical implications for the selection of prime boost combinations that are able to synergise. For example, it is clear that gene gun and i.n. vaccination with HBcAg and LT as an adjuvant maximise the $CD4^+$ response generated. Conversely, the

same boost preceded by an i.m. DNA vaccination led to a reduced $CD4^+$ response in the local lymph nodes.

The localisation of responses observed could be could be due to timing. It may be, for example, that the response progresses more rapidly following i.m. DNA vaccination than by gene gun, and at a later point after gene gun vaccination the lymph node T cells would also have migrated to the spleen. This could easily be investigated by a series of time course experiments.

It is also possible that the different responses observed are due to the dominance of different populations of T cells in the mice at the time at which responses were examined. It is known that naïve T cells migrate through lymphoid tissues but memory T cells are able to recirculate through non-lymphoid tissues such as the mucosa and skin (Dutton *et al.* 1998; Mackay and von Andrian 2001). There are two distinct subsets of these memory T cells following vaccination or infection; T central memory (Tcm) and T effector memory cells (Tem). Tcm are characterised by the ability to proliferate readily in response to antigen recognition, whereas Tem are more rapidly stimulated to effector function (Sallusto *et al.* 2004). As well as different functional capabilities, these populations are maintained in distinct areas. Tem, which are more short lived, are present in the non-lymphoid tissues to a greater extent, whereas the Tcm, which provide longer term memory, are found largely in the lymphoid tissues (Reinhardt and Jenkins 2003; Sallusto *et al.* 2004). It could be that Tcm and Tem are dominant in different tissues at the time point examined, and their relative ability to proliferate on restimulation gave rise to the results observed. The nature of the T cells involved could be evaluated in this context by FACS analysis of cell surface markers peculiar to each subset.

The above ideas may explain the situation in terms of the recent literature. However, perhaps a more obvious theory is that although activated T cells migrate

through non-lymphoid tissues (as has been previously observed; (Sallusto *et al.* 1999), the lymphoid tissues to which they home are restricted to those where they were first activated. This would make sense for protection against disease, since the locus of infection is likely to be close to where antigen is encountered, and in the longer term, re-infection is also likely to occur at the same location. Such localised T cells might be a subset of the Tcm or Tem population.

There is some evidence for this in the literature; memory T cells isolated from the gut were found to migrate preferentially back to the gut following activation and trafficked poorly through the skin (Mackay *et al.* 1992). However, little other work to date has focussed on localisation other than lymphoid/non-lymphoid. The migration of antigen-specific T cells could be investigated through the use of peptide-MHC tetrameters bound to fluorescent markers. These can bind to peptide-specific T cells via the TCR and thus be used to track cells (Reinhardt and Jenkins 2003).

8.6 Prophylactic and Therapeutic Vaccination

The heterologous vaccine strategies examined in this thesis show some promise for further development as vaccines to prevent or treat infectious disease. Although this body of work has concentrated on HBcAg, this as been used as a model antigen, and many interesting observations on the dynamics of DNA delivery and in particular prime-boost vaccination regimes could offer protection against a wide range of infectious disease or life threatening illnesses.

In humans and animal models, chronic HBV infection requires a powerful and broad Th1 immune response, incorporating strong cellular and humoral responses, in order to clear the virus. Therapeutic agents such as ribavirin and

recombinant IFN are believed to function by inducing Th1 responses (Hultgren *et al.* 1998), but they are of limited efficacy. Therapeutic vaccination provides another potential method of inducing the immune response required (Michel *et al.* 2001). At this stage it is difficult to assess which of the vaccination strategies examined in this thesis would be most suitable to take forward into the clinic in terms of potential success. Intramuscular DNA vaccination followed by an intramuscular protein boost would probably be the most appropriate without making alterations to the vaccination regime; this strategy gave $CD4^+$ and $CD8^+$ T cell responses together with an antibody response indicative of Th1 immunity and the magnitude of responses was enhanced following a heterologous boost. I.m. DNA vaccination followed by a heterologous boost, such as i.m. protein, may therefore act as a potentially effective therapeutic vaccine. However, the problems associated with intramuscular delivery of a vaccine candidate (even DNA), as outlined in the Introduction (section *), such as dangers of contamination of hypodermic needles and failure of uptake in the population, would still be present.

A possible solution to some of these problems would be the use of the novel gene gun technologies with intradermal delivery of DNA on gold particles. It has been shown in the past that gene gun vaccination results in strongly polarised Th2 responses, and as such would not be suitable for a vaccine designed to elicit Th1 immunity. However, results in this thesis suggest that the Th2 response is not as strongly biased when using a particulate antigen as in previous work with non-particulate antigen (see Chapter 5, Discussion section 8.2). This, coupled with other strategies for inducing Th1 cells by gene gun vaccination, such as co-delivery of CpG DNA (Schirmbeck and Reimann 2001; Zhou *et al.* 2003), could therefore also provide the basis for a therapeutic vaccine. There may also be potential in gene gun DNA vaccination followed by an intranasal boost. Although this strategy was not

successful at raising a mucosal response, this is not required in a therapeutic vaccine, since the infection is already established. Antibody responses were also low, but using higher doses of antigen or adjuvant may give higher responses.

As the majority of pathogens invade over mucosal surfaces, both i.m. and gene gun delivered DNA followed by a parenteral boost with HBcAg protein will not be able to generate the kind of mucosal immune responses required for protection. The introduction of a mucosal boost within the immunisation regime might produce such responses. This raises an additional problem; in the mucosal boosted experiments undertaken in this thesis, the mucosal adjuvant LT was used. Although LT is a potent mucosal adjuvant, it is highly toxic in humans and therefore cannot be licensed as an adjuvant. However, it may be possible that detoxified versions of the protein will be effective and safe (Pizza *et al.* 2001). Other strategies to incorporate a mucosal delivery of a boost in a prime-boost regime may offer a solution to this dilemma, such as the use of the live attenuated *Salmonella* delivering antigen utilised in this study. The *Salmonella* boost regime helped raise strong systemic antibody titres, which may indicate the presence of an effective $CD4^+$ T helper response. Mucosal responses were poor, and modifications to the vaccination regime would have to be made before this strategy could be used to provide protection at the mucosal surfaces. However, the antibody response in this case again indicated the dominance of a Th 1 phenotype after boosting, and since DNA vaccination alone can induce CTL responses, this strategy might also be effective clinically as a therapeutic vaccine.

REFERENCES

Abbas, A. K., K. M. Murphy and A. Sher (1996). "Functional diversity of helper T lymphocytes." Nature 383(6603): 787-793.

Ackerman, A. L. and P. Cresswell (2004). "Cellular mechanisms governing cross-presentation of exogenous antigens." Nature Immunology 5(7): 678-684.

Akbari, O., N. Panjwani, S. Garcia, R. Tascon, D. Lowrie and B. Stockinger (1999). "DNA Vaccination: Transfection and Activation of Dendritic Cells as Key Events for Immunity." Journal of Experimental Medicine 189(1): 169-178.

Armijos, R. X., M. M. Weigel, M. Calvopina, A. Hidalgo, W. Cevallos and J. Correa (2004). "Safety, immunogenecity, and efficacy of an autoclaved Leishmania amazonensis vaccine plus BCG adjuvant against New World cutaneous leishmaniasis." Vaccine 22(9-10): 1320-1326.

Attridge, S. R., R. Davies and J. T. LaBrooy (1997). "Oral delivery of foreign antigens by attenuated Salmonella: consequences of prior exposure to the vector strain." Vaccine 15(2): 155-162.

Bajenoff, M. and S. Guerder (2003). "Homing to Nonlymphoid Tissues Is Not Necessary for Effector Th1 Cell Differentiation." Journal of Immunology 171(12): 6355-6362.

Baldrick, P., D. Richardson, G. Elliott and A. W. Wheeler (2002). "Safety Evaluation of Monophosphoryl Lipid A (MPL): An Immunostimulatory Adjuvant." Regulatory Toxicology and Pharmacology 35(3): 398-413.

Bao, J. X. and J. D. Clements (1991). "Prior immunologic experience potentiates the subsequent antibody response when Salmonella strains are used as vaccine carriers." Infection and Immunity 59(10): 3841-3845.

Barrington, R., M. Zhang, M. Fischer and M. C. Carroll (2001). "The role of complement in inflammation and adaptive immunity." Immunological Reviews 180(1): 5-15.

Barry, M. and R. C. Bleackley (2002). "Cytotoxic T lymphocytes: all roads lead to death." Nature Reviews Immunology 2: 401-409.

Barry, M. A. and S. A. Johnston (1997). "Biological features of genetic immunization." Vaccine 15(8): 788-791.

Baumgarth, N. (2000). "A two-phase model of B-cell activation." Immunological Reviews 176: 171-80.

Baxby, D. (2002). "Smallpox vaccination techniques; from knives and forks to needles and pins." Vaccine 20(16): 2140-2149.

Bazin, H. (2003). "A brief history of the prevention of infectious diseases by immunisations." Comparative Immunology, Microbiology and Infectious Diseases 26(5-6): 293-308.

Bell, A. I., J. A. Cole and S. J. Busby (1990). "Molecular genetic analysis of an FNR-dependent anaerobically inducible Escherichia coli promoter." Molecular Microbiology 4(10): 1753-63.

Biron, C. A. (1998). "Role of early cytokines, including alpha and beta interferons (IFN-[alpha]\[beta]), in innate and adaptive immune responses to viral infections." Seminars in Immunology 10(5): 383-390.

Böcher, W. O., B. Dekel, W. Schwerin, M. Geissler, S. Hoffmann, A. Rohwer, F. Arditti, A. Cooper, H. Bernhard, A. Berrebi, S. Rose-John, Y. Shaul, P. R. Galle, H. F. Löhr and Y. Reisner (2001). "Induction of strong

hepatitis B virus (HBV) specific T helper cell and cytotoxic T lymphocyte responses by therapeutic vaccination in the trimera mouse model of chronic HBV infection." European Journal of Immunology **31**(7): 2071-2079.

Bohm, W., A. Kuhrober, T. Paier, T. Mertens, J. Reimann and R. Schirmbeck (1996). "DNA vector constructs that prime hepatitis B surface antigen-specific cytotoxic T lymphocyte and antibody responses in mice after intramuscular injection." Journal of Immunological Methods **193**(1): 29-40.

Bohm, W., T. Mertens, R. Schirmbeck and J. Reimann (1998). "Routes of plasmid DNA vaccination that prime murine humoral and cellular immune responses." Vaccine **16**(9-10): 949-954.

Bottcher, B., S. A. Wynne and R. A. Crowther (1997). "Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy." Nature **386**(6620): 88-91.

Brekke, O. H., T. E. Michaelsen and I. Sandlie (1995). "The structural requirements for complement activation by IgG: does it hinge on the hinge?" Immunology Today **16**(2): 85-90.

Bretscher, P. and M. Cohn (1970). "A theory of self-nonself discrimination." Science **169**(950): 1042-9.

Brodsky, F. M. and L. E. Guagliardi (1991). "The cell biology of antigen processing and presentation." Annual Review of Immunology **9**: 707-44.

Brown, W. C. and R. S. Corral (2002). "Stimulation of B lymphocytes, macrophages, and dendritic cells by protozoan DNA." Microbes and Infection 4(9): 969-974.

Bullas, L. R. and J. I. Ryu (1983). "Salmonella typhimurium LT2 strains which are r- m+ for all three chromosomally located systems of DNA restriction and modification." Journal of Bacteriology 156(1): 471-474.

Bumann, D., C. Hueck, T. Aebischer and T. F. Meyer (2000). "Recombinant live Salmonella spp. for human vaccination against heterologous pathogens." FEMS Immunology and Medical Microbiology 27(4): 357-364.

Carlson, S. A., M. Browning, K. E. Ferris and B. D. Jones (2000). "Identification of diminished tissue culture invasiveness among multiple antibiotic resistant Salmonella typhimurium DT104*1, *2." Microbial Pathogenesis 28(1): 37-44.

Carrol, M. E., P. S. Jackett, V. R. Aber and D. B. Lowrie (1979). "Phagolysosome formation, cyclic adenosine 3':5'-monophosphate and the fate of Salmonella typhimurium within mouse peritoneal macrophages." Journal of General Microbiology 110(2): 421-9.

Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia and M. Colonna (1999). "Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon." Nature Medicine 5(8): 919-923.

Chatfield, S., M. Roberts, P. Londono, I. Cropley, G. Douce and G. Dougan (1993). "The Development of Oral Vaccines Based on Live Attenuated Salmonella Strains." FEMS Immunology and Medical Microbiology 7(1): 1-7.

Chatfield, S. N., I. G. Charles, A. J. Makoff, M. D. Oxer, G. Dougan, D. Pickard, D. Slater and N. F. Fairweather (1992). "Use of the Nirb Promoter to Direct the Stable Expression of Heterologous Antigens in Salmonella

Oral Vaccine Strains - Development of a Single-Dose Oral Tetanus Vaccine." Bio-Technology 10(8): 888-892.

Chen, H. (2000). "Recent advances in mucosal vaccine development." Journal of Controlled Release 67(2-3): 117-128.

Cheng, L., P. Ziegelhoffer and N. Yang (1993). "In vivo Promoter Activity and Transgene Expression in Mammalian Somatic Tissues Evaluated by Using Particle Bombardment." Proceedings of the National Academy of Sciences of the United States of America 90(10): 4455-4459.

Chisari, F. V. and C. Ferrari (1995). "Hepatitis B virus immunopathogenesis." Annual Reviews in Immunology 13: 29-60.

Clark, M. R. (1997). "IgG effector mechanisms." Chemical Immunology 65: 88-110.

Cobo, E. R., D. Cano, O. Rossetti and C. M. Campero (2002). "Heifers immunized with whole-cell and membrane vaccines against Tritrichomonas foetus and naturally challenged with an infected bull." Veterinary Parasitology 109(3-4): 169-184.

Conway, J. F., N. Cheng, A. Zlotnick, P. T. Wingfield, S. J. Stahl and A. C. Steven (1997). "Visualisation of a 4-helix bundle in the hepatitis B virus capsid by cryo-electron microscopy." Nature 386: 91-94.

Corbeil, L. B. (2002). "Antibodies as effectors." Veterinary Immunology and Immunopathology 87(3-4): 169-175.

Correia-Neves, M., C. Waltzinger, D. Mathis and C. Benoist (2001). "The Shaping of the T Cell Repertoire." Immunity 14(1): 21-32.

Cowdery, J., J. Chace, A. Yi and A. Krieg (1996). "Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides." Journal of Immunology 156(12): 4570-4575.

Creusot, R. J., L. L. Thomsen, C. A. van Wely, P. Topley, J. P. Tite and B. M. Chain (2001). "Early commitment of adoptively transferred CD4+ T cells following particle-mediated DNA vaccination: implications for the study of immunomodulation." Vaccine 19(13-14): 1678-1687.

Culshaw, R., G. Bancroft and V. McDonald (1997). "Gut intraepithelial lymphocytes induce immunity against Cryptosporidium infection through a mechanism involving gamma interferon production." Infection and Immunity 65(8): 3074-3079.

Darji, A., S. zur Lage, A. I. Garbe, T. Chakraborty and S. Weiss (2000). "Oral delivery of DNA vaccines using attenuated Salmonella typhimurium as carrier." FEMS Immunology and Medical Microbiology 27(4): 341-349.

Davis, H. L. and M. J. McCluskie (1999). "DNA vaccines for viral diseases." Microbes and Infection 1(1): 7-21.

Davis, M. M. and P. J. Bjorkman (1988). "T-cell antigen receptor genes and T-cell recognition." Nature 334(6181): 395-402.

Devico, A. L., T. R. Fouts, M. T. Shata, R. Kamin-Lewis, G. K. Lewis and D. M. Hone (2002). "Development of an oral prime-boost strategy to elicit broadly neutralizing antibodies against HIV-1." Vaccine 20(15): 1968-1974.

Dockrell, D. H. (2003). "The multiple roles of Fas ligand in the pathogenesis of infectious diseases." Clinical Microbiology and Infection 9(8): 766-779.

Donnelly, J. J., J. B. Ulmer, J. W. Shiver and M. A. Liu (1997). "DNA vaccines." Annual Review of Immunology 15: 617-648.

Douce, G., M. Fontana, M. Pizza, R. Rappuoli and G. Dougan (1997). "Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin." Infect. Immun. 65(7): 2821-2828.

Dougan, G., M. Ghaem-Maghami, D. Pickard, G. Frankel, G. Douce, S. Clare, S. Dunstan and C. Simmons (2000). "The immune responses to bacterial antigens encountered in vivo at mucosal surfaces." Philosophical Transactions of the Royal Society of London 355(1397): 705-712.

Dunstan, S. J., C. P. Simmons and R. A. Strugnell (1998). "Comparison of the Abilities of Different Attenuated Salmonella typhimurium Strains To Elicit Humoral Immune Responses against a Heterologous Antigen." Infection and Immunity 66(2): 732-740.

Dunstan, S. J., C. P. Simmons and R. A. Strugnell (1999). "Use of In Vivo-Regulated Promoters To Deliver Antigens from Attenuated Salmonella enterica var. Typhimurium." Infection and Immunity 67(10): 5133-5141.

Dutton, R. W., L. M. Bradley and S. L. Swain (1998). "T Cell Memory." Annual Review of Immunology 16(1): 201-223.

Ehrenstein, M. R., T. L. O'Keefe, S. L. Davies and M. S. Neuberger (1998). "Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response." Proceedings of the National Academy of Sciences of the United States of America 95(17): 10089-10093.

Elmiyeh, B., I. S. Whitaker, M. J. James, C. A. Chahal, A. Galea and K. Alshafi (2004). "Needle-stick injuries in the National Health Service: a culture of silence." Journal of the Royal Society of Medicine 97(7): 326-7.

Epstein, J. E., Y. Charoenvit, K. E. Kester, R. Wang, R. Newcomer, S. Fitzpatrick, T. L. Richie, N. Tornieporth, D. G. Heppner and C. Ockenhouse (2004). "Safety, tolerability, and antibody responses in humans after sequential immunization with a PfCSP DNA vaccine followed by the recombinant protein vaccine RTS,S/AS02A." Vaccine 22(13-14): 1592-1603.

Eriksson, K. and J. Holmgren (2002). "Recent advances in mucosal vaccines and adjuvants." Current Opinion in Immunology **14**(5): 666-672.

Ernst, R. K., T. Guina and S. I. Miller (1999). "How Intracellular Bacteria Survive: Surface Modifications That Promote Resistance to Host Innate Immune Responses." Journal of infectious diseases **179**(S2): S326-30.

Ernst, R. K., T. Guina and S. I. Miller (2001). "Salmonella typhimurium outer membrane remodeling: role in resistance to host innate immunity." Microbes and Infection **3**(14-15): 1327-1334.

Estcourt, M. J., A. J. Ramsay, A. Brooks, S. A. Thomson, C. J. Medveckzy and I. A. Ramshaw (2002). "Prime-boost immunization generates a high frequency, high-avidity CD8(+) cytotoxic T lymphocyte population." International Immunology **14**(1): 31-7.

Ewing, W. H. (1972). "The nomenclature of Salmonella, its usage, and definitions for the three species." Canadian Journal of Microbiology **11**: 1629-37.

Feltquate, D., S. Heaney, R. Webster and H. Robinson (1997). "Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization." Journal of Immunology **158**(5): 2278-2284.

Foynes, S., J. L. Holley, H. S. Garmory, R. W. Titball and N. F. Fairweather (2003). "Vaccination against type F botulinum toxin using attenuated Salmonella enterica var Typhimurium strains expressing the BoNT/F HC fragment." Vaccine **21**(11-12): 1052-1059.

Freudenberg, M. A., T. Merlin, M. Gumenscheimer, C. Kalis, R. Landmann and C. Galanos (2001). "Role of lipopolysaccharide susceptibility in the innate immune response to Salmonella typhimurium infection: LPS, a

primary target for recognition of Gram-negative bacteria." Microbes and Infection 3(14-15): 1213-1222.

Gallichan, W. and K. Rosenthal (1996). "Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization." Journal of Experimental Medicine 184(5): 1879-1890.

Ganem, D. and R. Schneider (2001). Hepadnaviridae: the viruses and their replication. Field's virology. K. D. and P. Howley. Philadelphia, Lippincott-Raven: 2923-2970.

Garboczi, D. N., P. Ghosh, U. Utz, Q. R. Fan, W. E. Biddison and D. C. Wiley (1996). "Structure of the complex between human T-cell receptor, viral peptide and HLA-A2." Nature 384(6605): 134-41.

Goldrath, A. W. and M. J. Bevan (1999). "Selecting and maintaining a diverse T-cell repertoire." Nature 402(6759): 255-262.

Gonzalo, J.-A., T. Delaney, J. Corcoran, A. Goodearl, J. C. Gutierrez-Ramos and A. J. Coyle (2001). "Cutting Edge: The Related Molecules CD28 and Inducible Costimulator Deliver Both Unique and Complementary Signals Required for Optimal T Cell Activation." Journal of Immunology 166(1): 1-5.

Guidotti, L. G., T. Ishikawa, M. V. Hobbs and e. al. (1996). "Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes." Immunity 4(1): 25-36.

Guidotti, L. G., R. Rochford, J. Chung, M. Shapiro, R. Purcell and F. V. Chisari (1999). "Viral Clearance Without Destruction of Infected Cells During Acute HBV

Infection." Science 284((5415)): 825-829.

Harding, C. and R. Song (1994). "Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules." Journal of Immunology **153**(11): 4925-4933.

Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet and J. P. Allison (1992). "CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones." Nature **356**(6370): 607-9.

Hebeis, B. J., K. Klenovsek, P. Rohwer, U. Ritter, A. Schneider, M. Mach and T. H. Winkler (2004). "Activation of Virus-specific Memory B Cells in the Absence of T Cell Help." Journal of Experimental Medicine **199**(4): 593-602.

Heemels, M. T. and H. Ploegh (1995). "Generation, Translocation, and Presentation of MHC Class I-Restricted Peptides." Annual Review of Biochemistry **64**(1): 463-491.

Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton and D. W. Holden (1995). "Simultaneous Identification of Bacterial Virulence Genes by Negative Selection." Science **269**(5222): 400-403.

Herbert, W. J., P. C. Wilkinson and D. I. Stott (1995). The Dictionary of Immunology. London, AP.

Hohlfeld, R. and A. G. Engel (1994). "The immunobiology of muscle." Immunology Today **15**(6): 269-274.

Hormaeche, C. (1979). "Natural resistance to Salmonella typhimurium in different inbred mouse strains." Immunology **37**: 311.

Huang, Y., G. Hajishengallis and S. M. Michalek (2001). "Induction of Protective Immunity against Streptococcus mutans Colonization after Mucosal Immunization with Attenuated Salmonella enterica Serovar

Typhimurium Expressing an S. mutans Adhesin under the Control of In Vivo-Inducible nirB Promoter." Infection and Immunity 69(4): 2154-2161.

Hultgren, C., D. R. Milich, O. Weiland and M. Sallberg (1998). "The antiviral compound ribavirin modulates the T helper (Th)1/Th2 subset balance in hepatitis B and C virus-specific immune responses." Journal of General Virology 79: 2381-2391.

Imam, I. Z. (1981). "Field trial on the efficacy of oral Sabin vaccine versus Salk polio vaccine." Developments in biological Standardization 47: 215-221.

Janeway, C. A. and R. Medzhitov (2002). "Innate Immune Recognition." Annual Review of Immunology 20(1): 197-216.

Janeway, C. A., P. Travers, M. Walport and M. Schlomchik (2001). Immunobiology: the immune system in health and disease. Edinburgh, Garland.

Jenkins, M. K., A. Khoruts, E. Ingulli, D. L. Mueller, S. J. McSorley, R. L. Reinhardt, A. Itano and K. A. Pape (2001). "In Vivo Activation of Antigen-Specific CD4 T Cells." Annual Review of Immunology 19(1): 23-45.

Johnson, S., W. D. Sypura, D. N. Gerding, S. L. Ewing and E. N. Janoff (1995). "Selective Neutralization of a Bacterial Enterotoxin by Serum Immunoglobulin A in Response to Mucosal Disease." Infection and Immunity 63(8): 3166-3173.

Jones, B., H. Paterson, A. Hall and S. Falkow (1993). "Salmonella typhimurium Induces Membrane Ruffling by a Growth Factor- Receptor-Independent Mechanism." Proceedings of the National Academy of Sciences of the United States of America 90(21): 10390-10394.

Kanellos, T. S., D. K. Byarugaba, P. H. Russell, C. R. Howard and C. D. Partidos (2000). "Naked DNA when co-administered intranasally with heat-labile enterotoxin of Escherichia coli primes effectively for systemic B- and T-cell responses to the encoded antigen." Immunology Letters 74(3): 215-220.

Khan, S. A., R. Stratford, T. Wu, N. Mckelvie, T. Bellaby, Z. Hindle, K. A. Sinha, S. Eltze, P. Mastroeni and D. Pickard (2003). "Salmonella typhi and S. typhimurium derivatives harbouring deletions in aromatic biosynthesis and Salmonella Pathogenicity Island-2 (SPI-2) genes as vaccines and vectors." Vaccine 21(5-6): 538-548.

Klinman, D. M. (2003). "CpG DNA as a vaccine adjuvant." Expert Reviews in Vaccines 2: 305-315.

Kovacsovics-Bankowski, M., K. Clark, B. Benacerraf and K. Rock (1993). "Efficient Major Histocompatibility Complex Class I Presentation of Exogenous Antigen Upon Phagocytosis by Macrophages." Proceedings of the National Academy of Sciences of the United States of America 90(11): 4942-4946.

Krieg, A. M. (2002). "From A to Z on CpG." Trends in Immunology 23(2): 64-65.

Krieg, A. M., A.-K. Yi and G. Hartmann (1999). "Mechanisms and therapeutic applications of immune stimulatory CpG DNA." Pharmacology & Therapeutics 84(2): 113-120.

Krug, A., A. Towarowski, S. Britsch, S. Rothenfusser, V. Hornung, R. Bals, T. Giese, H. Engelmann, S. Endres and a. Krieg et (2001). "Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce

high amounts of IL-12." European Journal Of Immunology 31(10): 3026-3037.

Kuhrober, A., H. P. Pudollek, K. Reifenberg, F. V. Chisari, H. J. Schlicht, J. Reimann and R. Schirmbeck (1996). "DNA immunization induces antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2(b) mice." Journal of Immunology 156(10): 3687-3695.

Kuhrober, A., J. Wild, H. P. Pudollek, F. V. Chisari and J. Reimann (1997). "DNA vaccination with plasmids encoding the intracellular (HBcAg) or secreted (HBeAg) form of the core protein of hepatitis B virus primes T cell responses to two overlapping Kb- and Kd-restricted epitopes." International Immunology 9(8): 1203-12.

Kumaraguru, U., R. J. D. Rouse, S. K. Nair, B. D. Bruce and B. T. Rouse (2000). "Involvement of an ATP-Dependent Peptide Chaperone in Cross-Presentation After DNA Immunization." Journal of Immunology 165(2): 750-759.

Kundig, T. M., M. F. Bachmann, C. DiPaolo, J. J. Simard, M. Battegay, H. Lother, A. Gessner, K. Kuhlcke, P. S. Ohashi, H. Hengartner and et al. (1995). "Fibroblasts as efficient antigen-presenting cells in lymphoid organs." Science 268(5215): 1343-7.

Lange, U. G., P. Mastroeni, J. M. Blackwell and C. B. Stober (2004). "DNA-Salmonella enterica serovar Typhimurium primer-booster vaccination biases towards T helper 1 responses and enhances protection against Leishmania major infection in mice." Infection and Immunity 72(8): 4924-8.

Lehner, P. J. and P. Cresswell (2004). "Recent developments in MHC-class-I-mediated antigen presentation." Current Opinion in Immunology 16(1): 82-89.

Lodmell, D. L., N. B. Ray, J. T. Ulrich and L. C. Ewalt (2000). "DNA vaccination of mice against rabies virus: effects of the route of vaccination and the adjuvant monophosphoryl lipid A (MPL(R))." Vaccine 18(11-12): 1059-1066.

Londono, L. P., S. Chatfield, R. W. Tindle, K. Herd, X. M. Gao, I. Frazer and G. Dougan (1996). "Immunisation of mice using Salmonella typhimurium expressing human papillomavirus type 16 E7 epitopes inserted into hepatitis B virus core antigen." Vaccine 14(6): 545-552.

Londono-Arcila, P., D. Freeman, H. Kleanthous, A. M. O'Dowd, S. Lewis, A. K. Turner, E. L. Rees, T. J. Tibbitts, J. Greenwood, T. P. Monath and M. J. Darsley (2002). "Attenuated Salmonella enterica Serovar Typhi Expressing Urease Effectively Immunizes Mice against Helicobacter pylori Challenge as Part of a Heterologous Mucosal Priming-Parenteral Boosting Vaccination Regimen." Infection and Immunity 70(9): 5096-5106.

Lutz, M. B., N. Kukutsch, A. L. J. Ogilvie, S. Ro[ss]ner, F. Koch, N. Romani and G. Schuler (1999). "An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow." Journal of Immunological Methods 223(1): 77-92.

Mackay, C. R., W. L. Marston, L. Dudler, O. Spertini, T. F. Tedder and W. R. Hein (1992). "Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells." European Journal of Immunology 22(4): 887-895.

Mackay, C. R. and U. H. von Andrian (2001). "Immunology: Memory T Cells--Local Heroes in the Struggle for Immunity." Science 291(5512): 2323-2324.

Maecker, H. T., S. A. Ghanekar, M. A. Suni, X.-S. He, L. J. Picker and V. C. Maino (2001). "Factors Affecting the Efficiency of CD8+ T Cell Cross-Priming with Exogenous Antigens." Journal of immunology **166**(12): 7268-7275.

Manthorpe, M., F. Cornefert-Jensen, J. Hartikka, J. Felgner, A. Rundell, M. Margalith and V. Dwarki (1993). "Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice." Human Gene Therapy **4**(4): 419-31.

Marcus, S. L., J. H. Brumell, C. G. Pfeifer and B. B. Finlay (2000). "Salmonella pathogenicity islands: big virulence in small packages." Microbes and Infection **2**(2): 145-156.

Masopust, D., V. Vezys, A. L. Marzo and L. Lefrancois (2001). "Preferential Localization of Effector Memory Cells in Nonlymphoid Tissue." Science **291**(5512): 2413-2417.

Mastroeni, P. and M. Sheppard (2004). "Salmonella infections in the mouse model: host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues." Microbes and Infection **6**(4): 398-405.

McCluskie, M. J., R. D. Weeratna, J. D. Clements and H. L. Davis (2001). "Mucosal immunization of mice using CpG DNA and/or mutants of the heat-labile enterotoxin of Escherichia coli as adjuvants." Vaccine **19**(27): 3759-3768.

McCluskie, M. J., R. D. Weeratna, P. J. Payette and H. L. Davis (2002). "Parenteral and mucosal prime-boost immunization strategies in mice with hepatitis B surface antigen and CpG DNA." FEMS Immunology and Medical Microbiology **32**(3): 179-185.

McShane, H. (2002). "Prime-boost immunization strategies for infectious diseases." Current opinion in Molecular Therapeutics 4(1): 23-27.

Medina, E. and C. A. Guzman (2000). "Modulation of immune responses following antigen administration by mucosal route." FEMS Immunology and Medical Microbiology 27(4): 305-311.

Medina, E. and C. A. Guzman (2001). "Use of live bacterial vaccine vectors for antigen delivery: potential and limitations." Vaccine 19(13-14): 1573-1580.

Medzhitov, R. and C. A. Janeway Jr. (1998). "Innate immune recognition and control of adaptive immune responses." Seminars in Immunology 10(5): 351-353.

Michaelsen, T. E., J. Kolberg, A. Aase, T. K. Herstad and E. A. Hoiby (2004). "The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of Neisseria meningitidis." Scandinavian Journal of Infectious Diseases 59(1): 34-39.

Michel, M.-L., S. Pol, C. Brechot and P. Tiollais (2001). "Immunotherapy of chronic hepatitis B by anti HBV vaccine: from present to future." Vaccine 19(17-19): 2395-2399.

Milich, D., A. McLachlan, A. Moriarty and G. Thornton (1987). "Immune response to hepatitis B virus core antigen (HBcAg): localization of T cell recognition sites within HBcAg/HBeAg." Journal of Immunology 139(4): 1223-1231.

Milich, D. R., M. Chen, F. Schodel, D. L. Peterson, J. E. Jones and J. L. Hughes (1997). "Role of B cells in antigen presentation of the hepatitis

B core." Proceedings of the National Academy of Sciences of the United States of America 94(26): 14648-14653.

Milich, D. R., M. K. Chen, J. L. Hughes and J. E. Jones (1998). "The secreted hepatitis B precore antigen can modulate the immune response to the nucleocapsid: A mechanism for persistence." Journal of Immunology 160(4): 2013-2021.

Milich, D. R. and A. McLachlan (1986). "The nucleocapsid of Hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen." Science 234: 1398-1401.

Milich, D. R., D. Peterson, J. Zhenh, J. Hughes, R. Wirtz and F. Schodel (1995). "The hepatitis nucleocapsid as a vaccine carrier moiety." Annals of the New York Academy of Sciences 754: 187-201.

Milich, D. R., M. Sallberg and T. Maruyama (1995). "The Humoral Immune-Response in Acute and Chronic Hepatitis-B Virus-Infection." Springer Seminars in Immunopathology 17(2-3): 149-166.

Milich, D. R., F. Schodel, J. L. Hughes, J. E. Jones and D. L. Peterson (1997). "The hepatitis B virus core and e antigens elicit different Th cell subsets: Antigen structure can affect Th cell phenotype." Journal of Virology 71(3): 2192-2201.

Miller, I. A., S. Chatfield, G. Dougan, L. Desilva, H. S. Joysey and C. Hormaeche (1989). "Bacteriophage P22 as a vehicle for transducing cosmid gene banks between smooth strains of *Salmonella typhimurium*: Use in identifying a role for *aroD* in attenuating virulent *Salmonella* strains." Molecular Genetics and Genomics 215: 312-316.

Mills, D. M. and J. C. Cambier (2003). "B lymphocyte activation during cognate interactions with CD4+ T lymphocytes: molecular dynamics and immunologic consequences." Seminars in Immunology 15(6): 325-329.

Moingeon, P., J. Haensler and A. Lindberg (2001). "Towards the rational design of Th1 adjuvants." Vaccine 19(31): 4363-4372.

Mollenkopf, H.-J., D. Groine-Triebkorn, P. Andersen, J. Hess and S. H. E. Kaufmann (2001). "Protective efficacy against tuberculosis of ESAT-6 secreted by a live Salmonella typhimurium vaccine carrier strain and expressed by naked DNA." Vaccine 19(28-29): 4028-4035.

Moorthy, V. S., E. B. Imoukhuede, S. Keating, M. Pinder, D. Webster, M. A. Skinner, S. C. Gilbert, G. Walraven and A. V. Hill (2004). "Phase 1 evaluation of 3 highly immunogenic prime-boost regimens, including a 12-month reboosting vaccination, for malaria vaccination in Gambian men." Journal of Infectious Diseases 189(12): 2213-9. Epub 2004 May 24.

Morel, P. A., D. Falkner, J. Plowey, A. T. Larregina and J. Falo, Louis D. (2004). "DNA immunisation: altering the cellular localisation of expressed protein and the immunisation route allows manipulation of the immune response." Vaccine 22(3-4): 447-456.

Moretta, L., M. C. Mingari, C. Bottino, D. Pende, R. Biassoni and A. Moretta (2003). "Cellular and molecular basis of natural killer and natural killer-like activity." Immunology Letters 88(2): 89-93.

Mwau, M., I. Cebere, J. Sutton, P. Chikoti, N. Winstone, E. G.-T. Wee, T. Beattie, Y.-H. Chen, L. Dorrell, H. McShane, C. Schmidt, M. Brooks, S. Patel, J. Roberts, C. Conlon, S. L. Rowland-Jones, J. J. Bwayo, A. J. McMichael and T. Hanke (2004). "A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell

responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans." Journal of General Virology 85(4): 911-919.

Nambu, Y., M. Sugai, H. Gonda, C.-G. Lee, T. Katakai, Y. Agata, Y. Yokota and A. Shimizu (2003). "Transcription-Coupled Events Associating with Immunoglobulin Switch Region Chromatin." Science 302(5653): 2137-2140.

Neirynck, S., T. Deroo, X. Saelens, P. Vanlandschoot, W. Min Jou and W. Fiers (1999). "A universal influenza A vaccine based on the extracellular domain of the M2 protein." Nature Medicine 5(10): 1157-1163.

Nemazee, D. (2000). "Receptor selection in B and T lymphocytes." Annual Review of Immunology 18: 19-51.

O'Callaghan, D., D. J. Maskell, F. Y. Liew, C. S. F. Easmon and G. Dougan (1988). "Characterisation of aromatic and purine dependent Salmonella typhimurium: attenuation, persistence, and ability to induce protective immunity in BALB/c mice." Infection and Immunity 56(2): 419-423.

Ochsenbein, A. F., D. D. Pinschewer, S. Sierro, E. Horvath, H. Hengartner and R. M. Zinkernagel (2000). "Protective long-term antibody memory by antigen-driven and T help-dependent differentiation of long-lived memory B cells to short-lived plasma cells independent of secondary lymphoid organs." Proceedings of the National Academy of Sciences of the United States of America 97(24): 13263-13268.

Oltz, E. M. (2001). "Regulation of antigen receptor gene assembly in lymphocytes." Immunologic Research 32(2-3): 121-33.

Oxer, M. D., C. M. Bentley, J. G. Doyle, T. C. Peakman, I. G. Charles and A. J. Makoff (1991). "High-Level Heterologous Expression in Escherichia-Coli Using the Anaerobically-Activated Nirb Promoter." Nucleic Acids Research 19(11): 2889-2892.

Patrick, S. and M. J. Larkin (1995). Immunological aspects of bacterial virulence. Chichester, John Wiley and Sons.

Peiser, M., R. Wanner and G. Kolde (2004). "Human epidermal Langerhans cells differ from monocyte-derived Langerhans cells in CD80 expression and in secretion of IL-12 after CD40 cross-linking." Journal of Leukocyte Biology 76(3): 616-622.

Pieters, J. (1997). "MHC class II restricted antigen presentation." Current Opinion in Immunology 9(1): 89-96.

Pizza, M., M. M. Giuliani, M. R. Fontana, E. Monaci, G. Douce, G. Dougan, K. H. G. Mills, R. Rappuoli and G. Del Giudice (2001). "Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants." Vaccine 19(17-19): 2534-2541.

Plotkin, S. A. (2003). "Vaccines, Vaccination, and Vaccinology." Journal of Infectious Diseases 187: 1349-1359.

Prud'homme, G. J., B. R. Lawson, Y. Chang and A. N. Theofilopoulos (2001). "Immunotherapeutic gene transfer into muscle." Trends in Immunology 22(3): 149-155.

Ramshaw, I. A. and A. J. Ramsay (2000). "The prime-boost strategy: exciting prospects for improved vaccination." Immunology Today 21(4): 163-165.

Rappuoli, R., M. Pizza, G. Douce and G. Dougan (1999). "Structure and mucosal adjuvanticity of cholera and Escherichia coli heat-labile enterotoxins." Immunology Today 20(11): 493-500.

Reinhardt, R. L. and M. K. Jenkins (2003). "Whole-body analysis of T cell responses." Current Opinion in Immunology 15(4): 366-371.

Rissoan, M.-C., V. Soumelis, N. Kadowaki, G. Grouard, F. Briere, R. d. W. Malefyt and Y.-J. Liu (1999). "Reciprocal Control of T Helper Cell and Dendritic Cell Differentiation." Science **283**(5405): 1183-1186.

Roberts, M., S. Chatfield, D. Pickard, J. Li and A. Bacon (2000). "Comparison of Abilities of Salmonella enterica Serovar Typhimurium aroA aroD and aroA htrA Mutants To Act as Live Vectors." Infection and Immunity **68**(10): 6041-6043.

Roberts, M., J. Li, A. Bacon and S. Chatfield (1998). "Oral Vaccination against Tetanus: Comparison of the Immunogenicities of Salmonella Strains Expressing Fragment C from the nirB and htrA Promoters." Infection and Immunity **66**(7): 3080-3087.

Robinson, J. H. and A. A. Delvig (2002). "Diversity in MHC class II antigen presentation." Immunology **105**(3): 252-262.

Rojas, R. and G. Apodaca (2002). "Immunoglobulin transport across polarized epithelial cells." Nature Reviews Molecular Cell Biology **3**: 944-956.

Rossol, S., G. Marinos, P. Caucci, M. V. Singer, R. Williams and N. Naoumov (1997). "Interlukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B." Journal of Clinical Investigation **99**(12): 3025-3033.

Roth, E. and H. Pircher (2004). "IFN-gamma Promotes Fas Ligand- and Perforin-Mediated Liver Cell Destruction by Cytotoxic CD8 T Cells." Journal of Immunology **172**(3): 1588-1594.

Royle, M. C. J., S. Totemeyer, L. C. Alldridge, D. J. Maskell and C. E. Bryant (2003). "Stimulation of Toll-Like Receptor 4 by Lipopolysaccharide During Cellular Invasion by Live Salmonella typhimurium Is a Critical But Not

Exclusive Event Leading to Macrophage Responses." Journal of Immunology 170(11): 5445-5454.

Salerno-Goncalves, R., T. L. Wyant, M. F. Pasetti, M. Fernandez-Vina, C. O. Tacket, M. M. Levine and M. B. Sztein (2003). "Concomitant Induction of CD4+ and CD8+ T Cell Responses in Volunteers Immunized with Salmonella enterica Serovar Typhi Strain CVD 908-htrA." Journal of Immunology 170(5): 2734-2741.

Sallusto, F., D. Enig, R. Forster, M. Lipp and A. Lanzavecchia (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature 401: 708-712.

Sallusto, F., J. Geginat and A. Lanzavecchia (2004). "Central Memory and Effector Memory T Cell Subsets: Function, Generation, and Maintenance." Annual Review of Immunology 22(1): 745-763.

Sambrook, Fritsch and Maniatis (1989). Molecular Cloning, a laboratory guide, Cold Spring Harbor laboratory press.

Schirmbeck, R. and J. Reimann (2001). "Modulation of gene-gun-mediated Th2 immunity to hepatitis B surface antigen by bacterial CpG motifs or IL-12." Intervirology 44(2-3): 115-23.

Schodel, F., S. M. Kelly, D. L. Peterson, D. R. Milich and R. Curtiss (1994). "Hybrid Hepatitis-B Virus Core-Pre-S Proteins Synthesized in Avirulent Salmonella-Typhimurium and Salmonella-Typhi for Oral Vaccination." Infection and Immunity 62(5): 1669-1676.

Sedegah, M., T. R. Jones, M. Kaur, R. Hedstrom, P. Hobart, J. A. Tine and S. L. Hoffman (1998). "Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine."

Proceedings of the National Academy of Sciences of the United States of America 95(13): 7648-53.

Seki, N., A. D. Brooks, C. R. D. Carter, T. C. Back, E. M. Parsoneault, M. J. Smyth, R. H. Wiltrout and T. J. Sayers (2002). "Tumor-Specific CTL Kill Murine Renal Cancer Cells Using Both Perforin and Fas Ligand-Mediated Lysis In Vitro, But Cause Tumor Regression In Vivo in the Absence of Perforin." Journal of Immunology 168(7): 3484-3492.

Shedlock, D. J. and H. Shen (2003). "Requirement for CD4 T Cell Help in Generating Functional CD8 T Cell Memory." Science 300(5617): 337-339.

Stavnezer, J. (1996). "Immunoglobulin class switching." Current Opinion in Immunology 8(2): 199-205.

Stober, D., Z. Trobonjaca, J. Reimann and R. Schirmbeck (2002). "Dendritic cells pulsed with exogenous hepatitis B surface antigen particles efficiently present epitopes to MHC class I-restricted cytotoxic T cells." European Journal of Immunology 32(4): 1099-108.

Strid, J., J. Hourihane, I. Kimber, R. Callard and S. Strobel (2004). "Disruption of the stratum corneum allows potent epicutaneous immunization with protein antigens resulting in a dominant systemic Th2 response." European Journal of Immunology 34(8): 2100-2109.

Strugnell, R., G. Dougan, S. Chatfield, I. Charles, N. Fairweather, J. Tite, J. L. Li, J. Beesley and M. Roberts (1992). "Characterization of a Salmonella typhimurium aro vaccine strain expressing the P.69 antigen of Bordetella pertussis." Infection and Immunity 60(10): 3994-4002.

Sun, J. C. and M. J. Bevan (2003). "Defective CD8 T Cell Memory Following Acute Infection Without CD4 T Cell Help." Science 300(5617): 339-342.

Tagawa, S. T., P. Lee, J. Snively, W. Boswell, S. Ounpraseuth, S. Lee, B. Hickingbottom, J. Smith, D. Johnson and J. S. Weber (2003). "Phase I study of intranodal delivery of a plasmid DNA vaccine for patients with Stage IV melanoma." Cancer 98(1): 144-54.

Takeda, K. and S. Akira (2001). "Roles of Toll-like receptors in innate immune responses." Genes Cells 6(9): 733-42.

Tang, D. C., M. DeVit and S. A. Johnston (1992). "Genetic immunization is a simple method for eliciting an immune response." Nature 356(6365): 152-4.

Tighe, H., M. Corr, M. Roman and E. Raz (1998). "Gene vaccination: plasmid DNA is more than just a blueprint." Immunology Today 19(2): 89-97.

Tokuyama, H. and Y. Tokuyama (1999). "The Regulatory Effects of All-trans-Retinoic Acid on Isotype Switching: Retinoic Acid Induces IgA Switch Rearrangement in Cooperation with IL-5 and Inhibits IgG1 Switching." Cellular Immunology 192(1): 41-47.

Watts, C. (2004). "The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules." Nature Immunology 5(7): 685-92.

WHO (1996). "Hepatitis B vaccine - making global progress." WHO EPI update.

Williams, D. B., A. Vassilakos and W.-K. Suh (1996). "Peptide presentation by MHC class I molecules." Trends in Cell Biology 6(7): 267-273.

Williams, R., S. A. Johnston, M. Riedy, M. DeVit, S. G. McElligott and J. C. Sanford (1992). "Introduction of foreign genes into tissues of living mice by

DNA-coated microprojectiles." Proceedings of the National Academy of Sciences of the United States of America 88: 2726-2730.

Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani and P. L. Felgner (1990). "Direct gene transfer into mouse muscle in vivo." Science 247(4949 Pt 1): 1465-8.

Woo, P. C. Y., L.-p. Wong, B.-j. Zheng and K.-y. Yuen (2001). "Unique immunogenicity of hepatitis B virus DNA vaccine presented by live-attenuated Salmonella typhimurium." Vaccine 19(20-22): 2945-2954.

Wood, N. and B. Thorley (2003). "Towards global poliomyelitis eradication: The successes and challenges for a developed country." Journal of Paediatric Child Health 39(9): 647-650.

Woodland, D. L. (2004). "Jump-starting the immune system: prime-boosting comes of age." Trends in Immunology 25(2): 98-104.

Wynne, S. A., R. A. Crowther and A. G. W. Leslie (1999). "The crystal structure of the human hepatitis B virus capsid." Molecular Cell 3: 771-780.

Yang, C.-F., T. Naguib, S.-J. Yang, E. Nasr, J. Jorba, N. Ahmed, R. Campagnoli, H. van der Avoort, H. Shimizu, T. Yoneyama, T. Miyamura, M. Pallansch and O. Kew (2003). "Circulation of Endemic Type 2 Vaccine-Derived Poliovirus in Egypt from 1983 to 1993." Journal of Virology 77(15): 8366-8377.

Zajac, A. J., K. Murali-Krishna, J. N. Blattman and R. Ahmed (1998). "Therapeutic vaccination against chronic viral infection: the importance of cooperation between CD4+ and CD8+ T cells." Current Opinion in Immunology 10(4): 444-449.

Zal, T., A. Volkmann and B. Stockinger (1994). "Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells

specific for a blood-borne self-antigen." The Journal of Experimental Medicine 180(6): 2089-2099.

Zhou, X., L. Zheng, L. Liu, L. Xiang and Z. Yuan (2003). "T Helper 2 Immunity to Hepatitis B Surface Antigen Primed by Gene-Gun-Mediated DNA Vaccination can be Shifted Towards T helper 1 Immunity by Codelivery of CpG Motif-Containing Oligodeoxynucleotides." Scandinavian Journal of Immunology 58(3): 350-357.

Zhu, W., C. E. Thomas and P. F. Sparling (2004). "DNA Immunization of mice with a plasmid encoding Neisseria gonorrhea PorB protein by intramuscular injection and epidermal particle bombardment." Vaccine 22(5-6): 661-670.

Zlei, M. (2002). *In vitro* and *in vivo* evaluation of two DNA vaccine candidates. Infection and Immunity. Glasgow, University of Glasgow.